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Nuclear DNA content, base composition, heterochromatin and rDNA in *Picea omorika* and *Picea abies*

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Abstract Two closely related spruces, *Picea abies and Picea omorika*, a Balkan paleoendemic species, often share habitats, yet never hybridize in nature. The present study adresses their characteristics such as nuclear DNA content, base composition, heterochromatin and rDNA pattern. The genome size of *P. abies* was 10% larger than that of *P. omorika* when assessed by flow cytometry, respectively 2C=37.2 pg and 33.8 pg; although when estimated as total chromosome length it was virtually the same. The heterochromatin Chromomycin-A (CMA)/DAPI fluorochrome banding patterns of both *P. abies* and *P. omorika* are given here for the first time. Simultaneous FISH (fluorescent in situ hybridization) using 18S-26S and 5S rDNA probes revealed 16 18S rDNA sites in *P. omorika*, 12 18S rDNA sites in *P. abies*, and a single 5S rDNA locus in both species. The genomes have about 41% GC. The number and position of CMA/DAPI bands and rDNA loci provide good chromosome markers to clarify the karyotypes of the two species.

Keywords *Picea* · Fluorochrome banding · Flow cytometry · In situ hybridization · 18S-5.8S-26S and 5S rDNA · Nucleolar organizer region

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Introduction

Picea omorika (Pancic) Purkyne is a Balkan paleoendemic, tertiary relictual species, taxonomically close to the common European spruce *Picea abies* (L.) Karst. Before the last glaciation period *P. omorika* occupied a large area in Europe. However, the present area of this species is limited to the middle course of the Drina River in South-West Serbia and South-East Bosnia (Lakusic and Medjedovic 1974). Although *P. omorika* is tolerant to low temperatures and drought, and the trees of this species grow in a large edaphic and altitudinal range (300–1600 m), actual natural populations are, for unknown reasons, restricted to rare sites representing refuges for the vegetation during the last glaciation period. However, *P. omorika* is cultivated throughout Europe as an ornamental species due to its elegant shape and its resistance to atmospheric pollution. In contrast, *P. abies* is less tolerant to drought and needs low temperatures and humid conditions, which can be found at higher altitudes of Southern Europe. Nevertheless, this species is more competitive than *P. omorika* and has colonised a large part of Europe.

Despite their actual geographical proximity, *P. omorika* and *P. abies* have a different evolutionary history and are presumably genetically differentiated. Indeed, although these two species are sympatric they never hybridize in nature. On the other hand, hybrids between *P. omorika* and both of the two American species, *Picea sitchensis* (Bong.) Carr. and *Picea mariana* (Mill.) B. S. P., are found to be viable. Hybridization also occurs between *P. abies* and the Euro-Asiatic species, *Picea obovata*. Ledeb. (Mikkola 1972). Therefore, comparison of the genomes of *P. omorika* and *P. abies* is particularly interesting at the nuclear and chromosome levels.

In both species, as in the entire family *Pinaceae* that is characterized by prominent karyotypic uniformity, the chromosome pairs are difficult to distinguish due to their similar morphology and size. The karyotype data concerning these two species are scarce; classical cytogenetical studies have been done on *P. omorika* (Lakusic and

Medjedovic 1974; Hizume 1988) and *P. abies* (Hizume 1988), but no morphometric data have been carried out. A comparative morpho-karyological study has been made for different populations of *P. abies* (Terasmaa 1975). More recently, a karyotype constructed with the help of molecular cytogenetic markers (Lubaretz et al. 1996) and Giemsa C-banding pattern (Köhler et al. 1996) have been given for *P. abies*.

In the present study, genome size and base composition of *P. abies* and *P. omorika* were compared using flow cytometry and classical karyo-morphometry. Banding patterns, revealed after GC-specific staining with Chromomycin A (CMA) and AT-specific staining with bisbenzimide Hoechst 33258, as well as fluorescent in situ hybridization (FISH) using 18S-5.8S-26S (18S-26S) and 5S rDNA probes, enabled recognition of some of the chromosome pairs.

Materials and methods

Plant material

The origin of the material is presented in Table 1. For cytological studies, seeds of both species were collected on trees from natural populations, parks or botanical gardens. For flow cytometry, fresh needles from five individual trees of each species were collected in both Arboreta, the Faculty of Forestry, University of Zagreb (Croatia), and in Orsay University Park (France).

Flow cytometry

DNA content and GC percentage were determined by flow cytometry according to Marie and Brown (1993). Nuclei in the filtrate were stained with different fluorochromes: ethidium bromide (EB, Sigma), a DNA intercalating dye used with RNAse; the GC-specific dye CMA (Serva) and the AT-specific dye bisbenzimide Hoechst 33342 (Aldrich); used at concentrations of 30, 50, and 5 µg/ml, respectively. The stained nuclei were analyzed with a cytofluorimeter EPICS V (Coulter Inc., Fla., USA). Total nuclear DNA content and GC percentage were evaluated by comparison with leaf DNA values of *Triticum aestivum* var. Triple Dirk (2C=30.9 pg, 43.7% GC) which was used as an internal standard. The 2C DNA values were calculated assuming a linear relationship between ethidium bromide fluorescence and nuclear DNA content. The GC percentages were calculated according to Godelle et al. (1993). Experiments were repeated five times, over a 2 year-long period. Generally, each set comprised five individuals, measured separately and with repetition.

Feulgen staining and karyotype analysis

Root tips were obtained from seedlings germinated on moist filter paper in glass Petri dishes. Meristems were pre-treated with 0.05% colchicine for 16 h at room temperature, then fixed in 3:1 ethanol-acetic acid at 4°C for 24–48 h.

For karyotype observation, chromosomes were stained following Feulgen and Rosenback (1924) after hydrolysis in 1 N HCl at 60°C for 12 min. Chromosome arm lengths were measured on photographs of five well-spread metaphase plates per population. Idiograms were drawn from mean values and chromosome types were determined according to Levan et al. (1964) and Schlarbaum and Tsuchiya (1984). Karyological features were evaluated as total chromosome length (TL), the relative length of each chromosome pair (100× chromosome length/TL), the Asymmetry index (AsI%= 100 X long arm/total chromosome length), and the ratio between the longest and the shortest chromosome pair (R).

Fluorochrome banding

For fluorochrome banding, root-tip meristems were hydrolysed in 45% acetic acid for 13 min at 60°C and squashed in a drop of 45% acetic acid. Coverslips were removed following the technique of Conger and Fairchild (1953) and the slides were air dried for 12 h minimum. Preparations were then stained with bisbenzimide Hoechst 33258 (Serva) according to Martin and Hesemann (1988) and CMA (Serva) according to Schweizer (1976) and Kondo and Hizume (1982), with minor modifications: the concentration of CMA was 0.2 mg/ml, the buffer solution was made with 5 mM $MgSO_4$ and the treatment with Distamycin was omitted.

rDNA in situ hybridization

The location and number of 18S-26 S and 5 S rDNA sites were determined by fluorescence in situ hybridization (FISH) following Heslop-Harrison et al. (1991), with slight modifications described by Cerbah et al. (1998). Double FISH was carried out with two DNA probes; (1) the 18S rDNA probe was a clone of a 4 kb *Eco*RI fragment containing a part of 18S, 5.8S and 26S rDNA together with intergenic spacers of *Arabidopsis thaliana* labeled with Digoxigenin –11-dUTP (Boehringer Mannheim) conjugated to fluorescein-isothiocyanate, and (2) the pTA 794 probe (Gerlach and Dyer 1980) was a clone containing a 410-bp *Bam*H1 fragment of 5S wheat rDNA labeled with Fluorored-11-dUTP (Amersham). The two probes were labeled by a polymerase chain reaction. Chromosomes were denaturated in 70% formamide-2 × SSC. About 0.4 to 1 µg/ml of each probe was added to the hybridization mixture. DAPI identified AT-rich chromosome regions when used as a counterstain after in situ hybridization.

Table 1 Origin of material

Species	Seedlings	Needles
P. omorika (Pancic) Purkyne	Near Visegrad, Bosnia (leg. S. Medjedovic)	Arboretum of Faculty of Foresty, Zagreb, Croatia
	Park of Orsay University, France	Park of Orsay University, France
P. abies (L.) Karst.	Slovenia, (leg. J. Bavcon)	Arboretum of Faculty of Foresty, Zagreb, Croatia
		Park of Orsay University, France

Silver staining

Silver staining was done according to the method of Howell and Black (1980) in order to determine the number of nucleoli and the activity of the 18S-5.8S-26S ribosomal genes The ratio (gelatine/$AgNO_3$) used for selective staining was 1/3. The time of incubation at 37°C with silver was adjusted between 40 s to 60 s.

Results

Genome size and base composition

Analysis of total DNA content showed that *P. abies* both from Zagreb and Orsay had a significantly higher DNA quantity than *P. omorika* (Table 2). It is noted that the total DNA content for both species was a little higher (but not statistically significant) in the Orsay samples than in the Zagreb samples (Table 2). No significant differences were found in GC% between the two species. Table 3 summarizes the results from five experiments over 2 years. The mean 2 C value for *P. abies* was 37.2 pg and for *P. omorika* 33.8 pg. The base composition was about 41% GC.

The estimation of the genome size given by the total chromosome length was 278 µm for *P. omorika* and 272 µm for *P. abies* (Tables 3–5). However, the genome size of *P. abies* measured by flow cytometry was 10% larger than that of *P. omorika*.

Karyotype analyses

The two species have the same chromosome number (2n=24) and very similar karyotypes. Morphometric data concerning these karyotypes are presented in Table 4 for *P. omorika* and in Table 5 for *P. abies*. Our results are slightly different from those obtained by Lakusic and Medjedovic (1974) for *P. omorika* (10 metacentric and 2 submetacentric pairs), and Hizume (1988) for *P. omorika* (9 metacentric, 2 meta-submetacentric and 1 submetacentric pair) and for *P. abies* (10 metacentric and 2 meta-submetacentric pairs). In our sample, at least two sub-

Table 2 Total 2 C DNA and GC% in *Picea* species from Zagreb and Orsay (results of a single experiment)

[a] n, number of measurements from five individuals
[b] SD, standard deviation

	Zagreb		Orsay	
	P. omorika	*P. abies*	*P. omorika*	*P. abies*
2C DNA (pg)	33.2	36.8	34.4	37.8
n[a]	25	26	6	11
SD	0.57	0.88	0.56	1.18
GC (%)	41.6	40.5	40.3	42.1

Table 3 Total DNA content, GC %, chromosome length and CMA bands in *Picea* species (overall means)

[a] Global mean values from both samples (Zagreb and Orsay) obtained from five experiments in different seasons; number of measurements from five individuals, *n*=38 for *P. omorika*, *n*= 76 for *P. abies*

Species	Total chromosome length (µm)	2C DNA[a] (pg)	GC[a] (%)	Number and type of CMA bands
P. omorika	278	33.8±0.2	41.7±0.02	24: 4 very large and 8 large intercalary, 4 middle sized intercalary and centromeric, and 6 thin centromeric and 2 thin intercalary bands
P. abies	272	37.2±0.3	40.6±0.05	20: 12 large intercalary, 2 middle sized and 6 thin centromeric bands

Table 4 Morphometric data of the *P. omorika* chromosome complement. TLC (Total Length of diploid chromosome set)=278 µm; AsI (Asymmetric index)=55%; R (Ratio between the longest and the shortest chromosome pair)=1.64; abbreviations: m=metacentric (r=1–1.3); m-sm=metacentric-submetacentric (r=1.3–1.7); sm=submetacentric (r>1.7)

[a] Values are means with the standard error in parantheses

Chromosome pair	Long arm (µm)	Short arm (µm)	Total length TL (µm)	Relative length RL	Arm ratio r	Chromosome type
1	7.43 (0.46)[a]	6.65 (0.11)	14.08	10.12	1.12	m
2	7.23 (0.09)	6.64 (0.33)	13.87	9.97	1.09	m
3	7.05 (0.14)	5.85 (0.16)	12.90	9.28	1.21	m
4	6.74 (0.08)	5.94 (0.18)	12.68	9.12	1.13	m
5	6.29 (0.13)	6.29 (0.15)	12.58	9.05	1.00	m
6	5.92 (0.14)	5.88 (0.18)	11.80	8.49	1.01	m
7	6.12 (0.31)	5.15 (0.25)	11.27	8.10	1.19	m
8	6.07 (0.21)	5.15 (0.25)	11.22	8.07	1.18	m
9	5.80 (0.14)	4.72 (0.10)	10.52	7.57	1.23	m
10	6.52 (0.11)	3.52 (0.17)	10.04	7.22	1.85	sm
11	5.26 (0.11)	4.21 (0.09)	9.47	6.81	1.25	m
12	5.73 (0.17)	2.85 (0.14)	8.58	6.17	2.01	sm

Table 5 Morphometric data of *P. abies* chromosome complement. TLC (Total Length of diploid chromosome set) =272 µm; AsI (Asymmetric index)=56%; R (Ratio between the longest and the shortest chromosome pair)=1.86; abbreviations: m = metacentric (r=1–1.3); m-sm=metacentric-submetacentric (r=1.3–1.7); sm=submetacentric (r>1.7)

[a] Values are means with the standard error in parentheses

Chromosome pair	Long arm (µm)	Short arm (µm)	Total length TL (µm)	Relative length RL	Arm ratio r	Chromosome type
1	7.60 (0.67)	7.12 (0.61)	14.72	10.81	1.07	m
2	7.22 (0.41)	6.05 (0.65)	13.27	9.75	1.19	m
3	6.70 (0.57)	6.44 (0.58)	13.14	9.65	1.04	m
4	6.68 (0.45)	5.73 (0.31)	12.41	9.11	1.17	m
5	6.74 (0.63)	5.32 (0.43)	12.06	8.86	1.27	m
6	6.35 (0.51)	5.29 (0.44)	11.64	8.55	1.20	m
7	5.61 (0.59)	5.47 (0.51)	11.08	8.14	1.03	m
8	6.73 (0.58)	3.83 (0.29)	10.56	7.76	1.76	sm
9	5.85 (0.45)	4.34 (0.42)	10.19	7.48	1.35	m-sm
10	5.81 (0.53)	4.19 (0.38)	10.00	7.34	1.39	m-sm
11	5.33 (0.41)	3.84 (0.31)	9.17	6.73	1.39	m-sm
12	5.33 (0.35)	2.60 (0.23)	7.93	5.82	2.05	sm

metacentric pairs were observed in both species: pairs 10 and 12 in *P. omorika*, and pairs 8 and 12 in *P. abies*, similar to the observations of Terasmaa (1975).

In *P. abies* three pairs (9, 10 and 11) were of the meta-submetacentric type (r=1.3–1.7). The chromosome length varied gradually from 14.08 µm to 8.58 µm for *P. omorika*, and from 14.72 µm to 7.93 µm for *P. abies*. The value of R (ratio between longest and shortest chromosome pair) was 1.64 for *P. omorika* and 1.86 for *P. abies*. The value of the asymmetry index (AsI) was very similar, 55% for *P. omorika* and 56% for *P. abies*.

Fluorochrome banding patterns and nucleolar organizer activity

The fluorochrome banding patterns of the two species investigated were different. In *P. abies* (Fig 1a), 20 CMA bands (12 large intercalary, two middle sized centromeric and six very thin centromeric bands) were observed and in *P. omorika* (Fig. 1e) 24 CMA bands (four very large and eight large intercalary, four middle-sized intercalary and centromeric, and eight thin centromeric and intercalary bands) appeared at intercalary and centromeric positions of certain chromosomes. The position of CMA bands is presented on the idiograms of *P. omorika* (Fig. 2a) and *P. abies* (Fig. 2b). In *P. abies*, intercalary bands were observed on six chromosome pairs, four pairs displayed centromeric bands and four pairs lacked any CMA band. In *P. omorika*, intercalary CMA bands appeared on eight chromosome pairs, three pairs were characterized by both centromeric and intercalary CMA bands, and the smallest pair by only a centromeric band, while no bands were seen on three pairs.

The large and middle-sized CMA bands appeared Hoechst-negative. DAPI revealed positive bands at the centromeric region of most of the chromosomes of both species, and several intercalary bands (Fig. 1b and f). When applied as a counterstain in FISH experiments (after denaturation of chromosomal DNA), DAPI produced more prominent banding patterns that differed for the two species. *P. omorika* possessed ten centromeric bands, two of them being characteristic for the smallest submetacentric chromosome pair (Fig. 1f). All other DAPI bands in this species were present in the centromeres of metacentric chromosomes of similar size and shape. The smallest chromosome pair in *P. abies* did not possess a centromeric DAPI band, as in *P. omorika*. Six of 12 DAPI bands in this species were in the centromeric region of four metacentric and two submetacentric chromosomes, and the other six DAPI bands were in intercalary regions of metacentric chromosomes. Intercalary DAPI bands were, at the same time, CMA positive and corresponded to 18S-26S and 5S rDNA sites (Fig. 1 b, c and d, arrows). Two metacentric pairs displayed DAPI bands in both centromeric and intercalary positions.

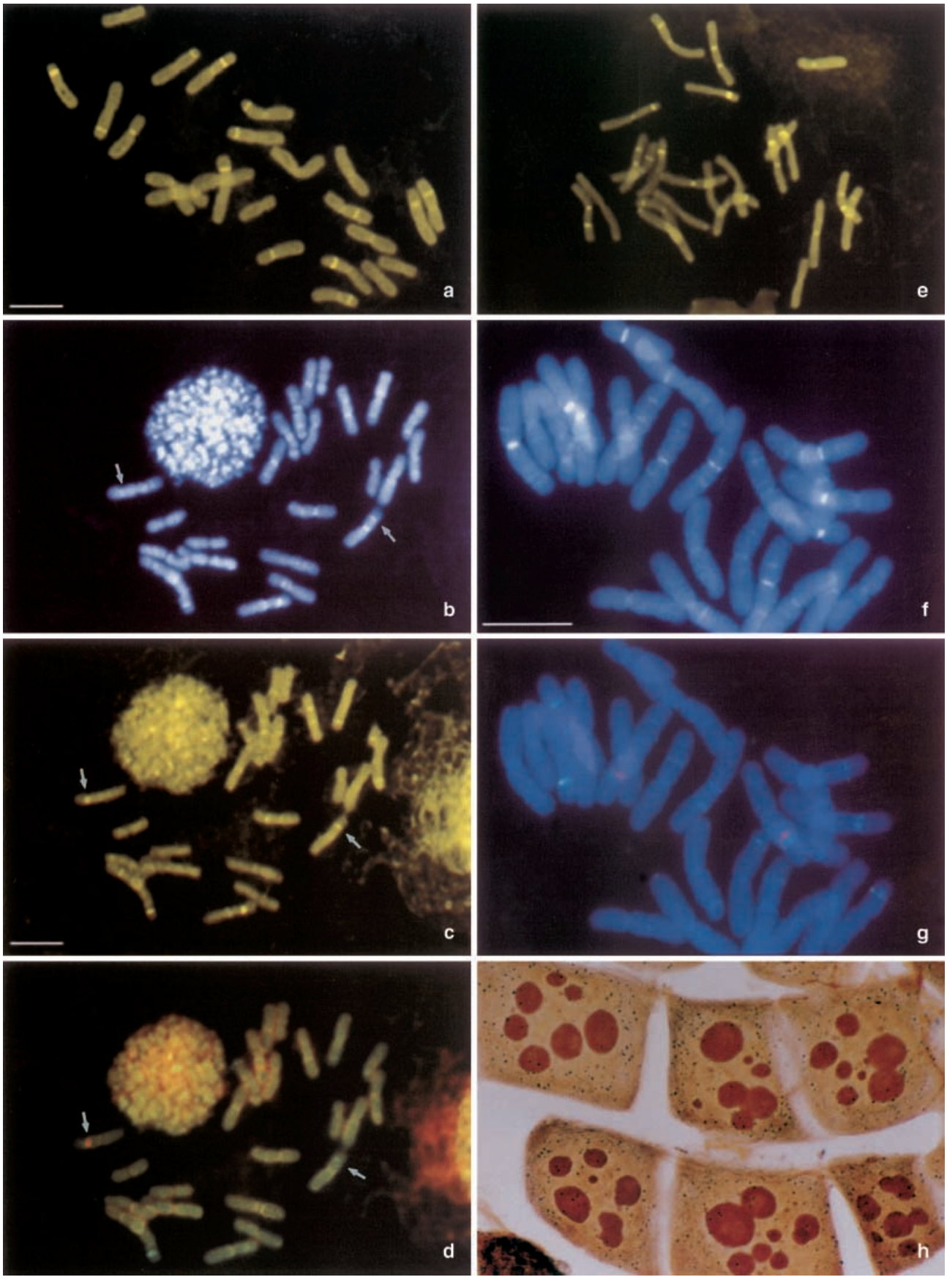
Numerous chromocenters stained with CMA (corresponding to GC-rich heterochromatic bands) were observed in the interphase nuclei in both species and nucleoli were evident as CMA-negative areas.

The number of $AgNO_3$-stained nucleoli per nucleus in *P. omorika* was generally six or eight (Fig. 1h) while the maximum number was 16 (Fig. 3a). In *P. abies* the mean number of nucleoli was six and the maximum number was 12 (Fig. 3b). The maximum number of nucleoli per species corresponded to the number of intercalary CMA bands located in the secondary constrictions (SCs).

Number and position of rDNA loci

FISH revealed 12 fluorescent signals on the chromosomes of *P. abies* (Fig. 1d) and 16 signals on the chromosomes of *P. omorika* (Fig. 1g) corresponding to 18S-26S rDNA sites, all in intercalary GC-rich regions. In both

Fig. 1 Fluorochrome banding of metaphase chromosomes in *P. abies* (**a** and **c** CMA; **b** DAPI) and in *P. omorika* (**e** CMA; **f** DAPI). FISH with 18S-26S (*green signals*) and 5S (*red signals*) rDNA probes in *P. abies* (**d**) and in *P. omorika* (**g**). Silver staining of nucleoli (**h**) in *P. omorika*. The 5S rDNA chromosomal site (*arrows* on Fig. 1d) stain positively with DAPI (*arrows* on Fig. 1b) and with CMA (*arrows* on Fig. 1c). Because of the co-localization of the 5S rDNA locus with one 18S rDNA locus, only one red signal was visible on Fig. 1d. *Bars*=10 µm ▶


a
e
b
f
c
g
d
h

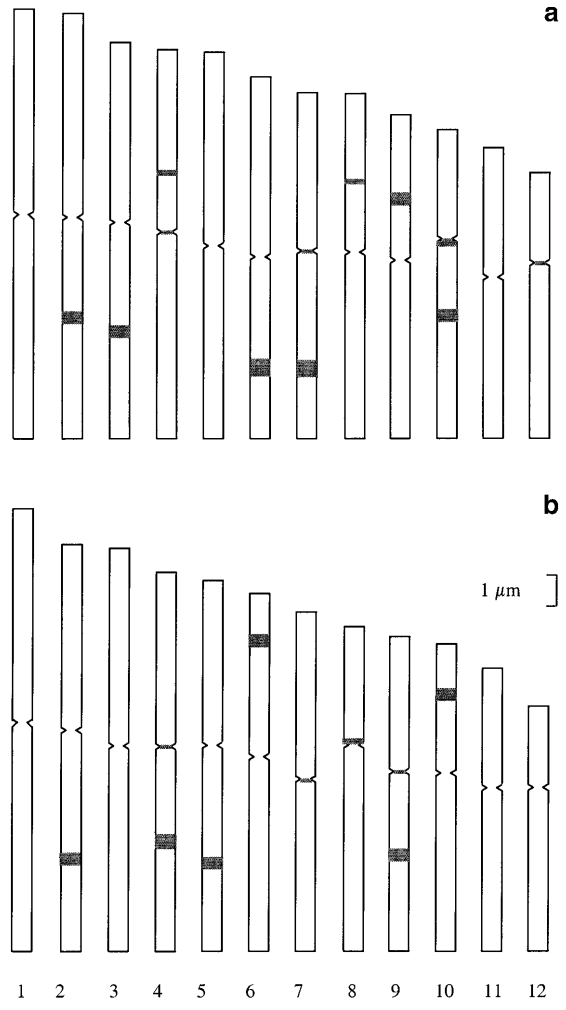


Fig. 2 Karyotypes of *P. omorika* (**a**) and *P. abies* (**b**) showing position of CMA bands

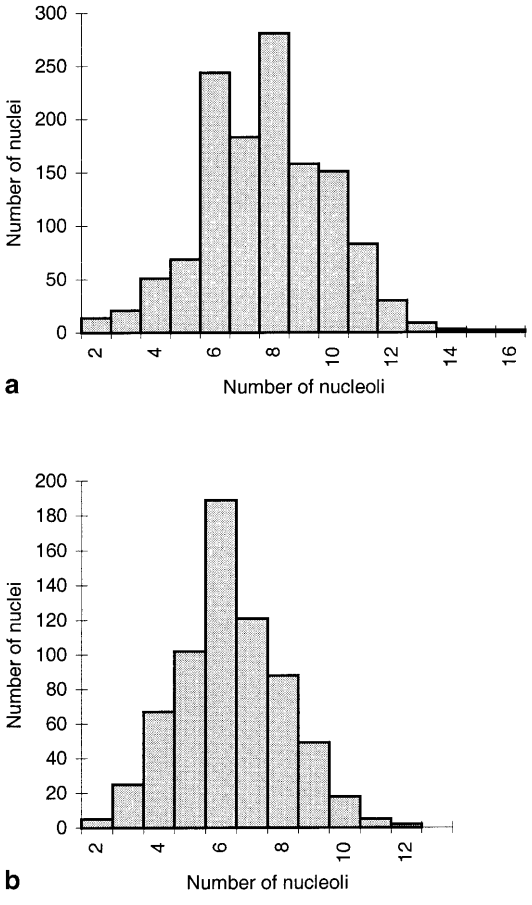


Fig. 3 Histogram of number of nucleoli per nucleus in *P. omorika* (**a**) and *P. abies* (**b**)

species, only one 5S rDNA locus was found, positioned near the 18S-26S locus on a metacentric chromosome pair. In *P. abies*, heterochromatin associated with the 5S rDNA site stained positively with DAPI and CMA (Fig. 1b and c, arrows). Because of the co-localization of the 5S rDNA locus with one 18S rDNA locus, it was difficult to see both signals at the same time, because of the overlapping of the green fluorescence (Fig. 1d, arrows).

Discussion

As for other conifer genomes, the 2C DNA content found for *P. abies* and *P. omorika* is quite high. These large genomes are very complex, and recently the contribution of minisatellites and telomeric sequences to this complexity has been demonstrated by Schmidt et al. (2000).

P. omorika has a 2C genome size of 33.8 pg, 10% smaller than *P. abies* when estimated by flow cytometry, but a similar genome size when estimated in terms of total chromosome length.

In different populations of *P. abies*, Terasmaa (1972, 1975) reported five chromosome pairs having a "linear satellite" and observed up to ten nucleoli. Our results rather agree with Hizume's (1988) finding of six chromosome pairs with secondary constrictions (SCs) and 2 to 12 nucleoli per cell. Indeed, we have found that the six NOR-bearing chromosome pairs contain the 18S-26S rDNA genes in their SCs and the same chromosomes are characterized by the large intercalary CMA_3 bands. Nuclei displayed maximally 12 nucleoli in agreement with the existence of six NOR-bearing chromosomes. Lubaretz et al. (1996) reported the same number of six 18S-5.8S-26S rDNA loci and one 5S rDNA locus, which was very close to the 18S locus of a metacentric chromosome pair.

In *P. omorika*, Hizume (1988) found a single chromosome pair with a SC, while Lakusic and Medjedovic (1974) reported three chromosome pairs bearing SCs. Our results show that at least six chromosome pairs have a SC corresponding to the large CMA bands. However, the maximum number of nucleoli was 16 corresponding to the same number of 18S-26S loci, which indicates that all rDNA sites can be active.

CMA banding patterns have already been reported for several species of the *Pinaceae*; the CMA pattern, reported for *Picea jezoensis* (Hizume et al. 1989), is simi-

lar to that of *P. omorika*. Twenty two bands were seen: 12 at the interstitial region in six chromosome pairs and ten at the proximal region in five chromosome pairs. Hybrids between these two species have been observed (Mikkola 1972) indicating their close relationship and similar chromosome organization. *Picea brachytyla* var. *complanata* has been characterized with 14 CMA bands: ten large bands at the interstitial regions of five chromosome pairs and four thin bands at the centromeric regions of two chromosome pairs (Hizume at al. 1991). Our preliminary study (Papes et al. 1997) of chromosome banding reported similar results for *P. abies*. In addition the present study identifies six thin centromeric bands.

Hizume et al. (1992), using a 18S rDNA probe, has found that the location of FISH signals corresponds to all interstitial CMA-bands in *Pinus densiflora* (14 bands) and in *Pinus thunbergii* (12 bands). In some other conifers more than ten rDNA sites were detected per diploid genome. For example, *Pinus radiata* has ten sites (Cullis et al. 1988), *Pinus sylvestris* 16 sites according to Karvonen et al. (1993) and 14 sites according to Lubaretz et al. (1996), *Pinus elliottii* 16 sites (Doudrick et al. 1995), *Picea glauca* 14 sites (Brown et al. 1993), *Picea sitchensis* ten sites of rDNA (Brown and Carlson 1997). Although only six sites of 18S rDNA were reported for *Larix decidua* by Lubaretz et al. (1996), it seems that an important number of rDNA loci (more than ten sites) is common among conifers, especially for the *Pinus* and *Picea* genera. The number of 16 rDNA sites is among the highest reported for diploid organisms. The evolutionary significance of such a high number of rDNA loci remains unclear.

In both species analysed in this study, nucleoli of different sizes were observed. This is another common feature of conifers, which is a result of the frequent fusion of nucleoli (MacPherson and Filion 1981; Karvonen et al. 1993). Karvonen et al. (1993) have observed associations between NORs in metaphase chromosomes of Scots pine and therefore deduced that several NOR-bearing chromosomes were involved in the formation of a large nucleolus at interphase.

The identification of chromosome pairs in conifers by conventional karyotype analysis is very difficult since chromosomes are similar in shape and size. The high number and the length of chromosomes is an additional problem for obtaining suitable metaphase spreads. Although more markers are desirable, we have shown in the present study that fluorochrome banding and in situ hybridization with rDNA probes provide good tools to clarify karyotypes of the two related species, *P. omorika* and *P. abies*. Even though we could not distinguish every chromosome pair, the chromosome organization has been found to be different for these two species. Although having a common localisation of 5S rDNA, they display a different genome size as well as a different number of CMA bands and rDNA sites. It will be interesting to compare the chromosome organization *of Picea mariana* and *Picea obovata*. These two geographically distant species hybridize naturally with *P. omorika* and *P. abies* respectively.

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