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Karyotype analysis reveals interspecific differentiation in the genus *Cedrus* despite genome size and base composition constancy

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Abstract The DNA content and GC% of the four true cedar (*Cedrus*) species, *C. atlantica*, *C. brevifolia*, *C. deodara* and *C. libani*, were assessed. Genome size was homogeneous among representative populations of the four species with an average of 32.6±0.6 pg per 2 C or 15.7×10^9 base pairs per 1 C. The composition in GC was calculated to be 40.7%. A simple monosomatic haploid level was found in the megagametophyte, as compared to the diploid level of the corresponding embryo. Cytogenetic studies showed a diploid chromosome number of 2n=2x=24 in 11 populations sampled over the four species. The chromosome complements have similar morphology and symmetry. However, fluorochromes revealed specific banding patterns in each of the four cedar species. Eight GC-rich chromomycin A_3 bands were observed in *Cedrus deodara* chromosomes, six in both *Cedrus libani* and *Cedrus brevifolia*, and four bands in *Cedrus atlantica* chromosomes. Moreover, Hoechst 33258 fluorochrome revealed AT-rich sequences specifically located in the centromeric regions while the GC-rich sequences appeared negatively stained. These investigations provide a systematic characterisation of the *Cedrus* genus and should contribute towards clarification of the phylogenetic relationships among the four species.

Keywords Base composition · *Cedrus* · Fluorochrome banding · Genome size · Karyotype analysis · Gymnosperm

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Introduction

The *Cedrus* Tree genus (*Pinaceae* Lindl.) is commonly regarded as comprising four species: *Cedrus atlantica* Manetti, *Cedrus brevifolia* Henry, *Cedrus deodara* Loudon and *Cedrus libani* Barrel (Vidakovic 1991). Phylogenetic relationships among the four species have been the subject of numerous debates (Davis 1965; Greuter et al. 1984). Discrimination among these four species has frequently been based on external morphology which may change according to biotic and abiotic factors leading to several different classifications. The *Cedrus* genus is divided into three species according to the Flora Europaea (Tutin et al. 1964), and more, since two Mediterranean species are treated as subspecies of *C. libani* in (Davis et al. 1965).

Based on paleobotanical evidence, true cedars appear to have been widely, but discontinuously, distributed in the Old World in the upper Tertiary (Fabre 1976). Extensive exploitation and climatic changes such as glaciations have reduced their habitat to their present geographical distribution. Cedar wood has been intensively used over centuries by Assyrians, Babylonians, Phoenicians and Egyptians in shipbuilding, temple decoration, mummification, embalming and resin production (Chaney and Basbous 1978).

C. libani, the Cedar of Lebanon, is native to Lebanon, Syria and Turkey (primarily in the Taurus Mountains). The renown of the species comes from its remarkable appearance and mythological and Biblical associations. *C. brevifolia*, the Cyprus cedar, is endemic to Cyprus Island and has also been considered as a variety of *C. libani* (Tutin et al. 1964). *C. atlantica*, the Atlas cedar, grows in the Atlas and Riff Mountains of North Africa, Morocco and Algeria. It is morphologically closely related to the

Cedar of Lebanon (Alptekin et al. 1997). *C. deodara*, the Himalayan cedar, occurs in the Himalayan Mountains and is an important timber tree in India. Due to its high adaptation to a large variety of environmental conditions, cedar has been successfully introduced in many *circum* – Mediterranean countries (Toth 1979).

Inter- and intra-specific genome size variation of conifers, and especially the *Pinaceae*, has been reported (Ohri and Khoshoo 1986; O'Brien et al. 1996; Murray 1998).

However, identification of an individual chromosome in conifers by conventional karyotype analysis has always been difficult since they are frequently metacentric and of similar size. Karyotypes of related species are poorly differentiated (Natarajan et al. 1961; Pederick 1970; Saylor 1972). Recently distinctive karyotype analysis of *Pinaceae* species (2n=2x=24) has been established through fluorochrome banding techniques (Hizume et al. 1983; Brown et al. 1993; Doudrick et al. 1995; Papes et al. 1997).

Despite its economical and ecological value, little is known about the *Cedrus* genus other than its elementary botany. Genome-size determination and karyotype analysis using fluorescence in situ hybridization, which are useful parameters for ecophysiology, molecular biology and systematic evolutionary studies, have not been conducted for this genus. This paper for the first time presents comprehensive data on genome size, base composition and cytogenetical analysis using fluorochrome banding for the genus *Cedrus*.

Materials and methods

Plant material

Eleven *Cedrus* populations were sampled for this study (Table 1). Seeds were extracted from mature, open-pollinated cones and bulked. The seeds were stored at 4°C until used for study. Seeds from each population were used for the genome size, base composition and cytological analysis.

Flow cytometry measurements

For assessment of ploidy level, DNA content and base composition by flow cytometry, we collected tissues from different parts of cedar plants, namely the root, stem and needles, as well as the embryo and endosperm, with the exception of the Bcharre Lebanese population where only needles were used. Seeds were first imbibed overnight and then dissected to separate the embryo from the endosperm (megagametophyte). *Pisum sativum* (2C=8.37 pg; 40.5% GC) and hexaploid wheat (2C=30.9 pg/2 C) were used as an internal standard (Marie and Brown 1993).

Four different buffers were tested in order to release nuclei from plant tissues (Galbraith et al. 1983; Dolezel et al. 1989; Ulrich and Ulrich 1991; Marie and Brown 1993). Cedar tissues were co-chopped with *P. sativum* or wheat leaves with a razor blade in Galbraith buffer (Galbraith et al. 1983) and slightly modified by the addition of 10 mM of metabisulfite, 1% (w/v) Triton X-100 and 1% (w/v) polyethylene glycol (PEG) 8000. Nuclei were then filtered through a 75-μm nylon filter in order to eliminate cell debris after the addition of 5 units/ml of RNase A (Roche, France) and 50 μg/ml of propidium iodide (Sigma-Aldrich, France). Samples were left for 30 min on ice before measurement.

Total 2 C DNA content was evaluated using the leaf 2 C DNA value of *P. sativum*, assuming a linear relationship between fluorescence ratio and the amount of DNA.

Genome base composition was determined using three fluorochromes according to the model developed by Godelle et al. (1993), which is based on a curvilinear relationship between the fluorescence intensity of the different fluorochromes and the ATs, and GCs contents. The quantum yield of Hoechst 33342 is highest when it is fixed on a succession of at least five AT whereas two, three or four consecutive GC can fix mithramycin. The fluorescence intensity of 2 C nuclei was assessed respectively with Hoechst 33342 (5 μg/ml, Aldrich) after staining for 3 min or mithramycin (50 μg/ml, Sigma; 55 mM $MgCl_2$; 1% formaldehyde) after 30 min. For each sample, measurements were made on 2500 nuclei with duplication. Base composition was calculated according to the Godelle formula (Godelle et al. 1993) with two independent estimates (Hoechst and mithramycin). The estimates were expressed in GC% units, and the resulting mean was corrected for any minor deviation of the sum (AT+GC) from 100%.

Fluorescence analysis of the stained nuclei was conducted on an Epics V cytometer (Beckman-Coulter, Roissy, France) with an argon laser at 488 nm for propidium iodide, 458 nm for mithramycin and 357 nm for Hoechst. The cytometer linearity was adjusted before each run set.

Cytological techniques

For germination, seeds were put on wet Whatman paper at 4°C for 28 days and then placed at 20°C for several days. Emerging root tips from 30 to 40 day old germinated seeds were used for cytogenetic investigations.

To observe metaphase plates, root-tip meristems were immersed in 0.05% (w/v) colchicine at room temperature for 19–

Table 1 Geographic origins of the 11 *Cedrus* populations sampled for this study. Seeds were collected by Dr. M. Bariteau, Unité de Recherches Forestières Méditerranéennes, INRA, avenue Vivaldi, 84000 Avignon, France (A); Dr. Neophyton, Forestry department Nicosia, Cyprus (B); Dr. Fikret Izik, Antalya, Turkey (C); and Dr. Zeki Kaya, Ankara, Turkey (D)

Species	Population/country	Seed source	Lat. °N	Long. °W	Alt. (m)
C. atlantica Manetti	Ouled-Yacoub/Algeria	A	35°20′	06°53′	1550
	Seheb/Morocco	A	33°21′	05°14′	1700–1800
	Tizi-Ifri/Morocco	A	34°52′	04°16′	1750
	Ventoux/France	A	44°07′	05°11′	780–930
C. brevifolia Henry	Paphos/Cyprus	B	34°52′	32°59′	1400–1600
C. deodara Loudon	Mirdeh/Afghanistan	A	35°	71°	2000
C. libani Barrel	Barouk/Lebanon	A	33°36′	35°41′	1500–1700
	Bcharre/Lebanon	A	34°47′	36°05′	1900
	Çamkuyusu/Turkey	C	36°35′	30°01′	1700–1900
	Çatalan/Turkey	D	40°47′	36°34′	1100
	Karaçay/Turkey	A	36°24′	29°26′	1550

Table 2 Nuclear DNA content and base composition of the 11 *Cedrus* populations sampled for this study. N, number of seeds analysed; SD, standard deviation; CV, coefficient of variation of the *Cedrus* 2C estimate; nd., not determined. The last two columns are derived from the preceding columns

Cedrus species	Origin	N	2C genome size (pg)	SD (pg)	CV (%)	AT		GC		AT+GC (%)	Mean corrected GC estimates (%)
						N	(%)	N	(%)		
C. atlantica	Ouled-Yacoub	6	32.5	0.17	0.52	10	59.4	20	40.4	99.8	40.6
	Seheb	5	32.2	0.35	1.08	12	59.2	6	40.6	99.8	40.8
	Tizil-Ifri	6	32.4	0.60	1.84	8	59.5	8	40.0	99.5	40.4
	Ventoux	8	32.5	0.52	1.58	12	59.2	10	40.3	99.5	40.7
C. atlantica species mean			32.4	0.20							40.5
C. brevifolia	Paphos	10	31.8	0.64	2.01	22	59.5	16	40.4	99.9	40.5
C. deodara	Mirdeh	6	32.6	0.40	1.22	23	59.1	20	40.4	99.5	40.8
C. libani	Barouk	6	32.0	0.43	1.34	11	59.1	6	40.5	99.6	40.9
	Bcharre	18	32.7	0.63	1.92	12	59.2	37	40.5	99.7	40.8
	Çamkuyusu	20	32.7	0.38	1.17		nd.	nd.	nd.		
	Çatalan	12	32.9	0.43	1.30	7	59.1	6	40.5	99.6	40.9
	Karaçay	6	32.9	0.16	0.50	8	58.8	6	40.0	98.8	40.9
C. libani species mean			32.5	0.41							40.9

24 h and then fixed in ethanol: acetic acid (3:1, v/v) at 4°C over 48 h, and finally stored in 70% ethanol at 4°C. Pre-treated root-tips were softened in an enzyme solution containing 1% (w/v) Pectolyase Y23 (Seishin corporation, Tokyo, Japan), 4% (w/v) cellulase R10 (Onozuka, Tokyo, Japan) and 4% (w/v) hemicellulase (Sigma-Aldrich, France) in 0.05 M citrate buffer, pH 4.5, at 37°C for approximately 1 h, the duration of this treatment depending upon the age of the root. The roots were then gently squashed in a drop of 45% acetic acid. After removal of the coverslips with liquid nitrogen, the slides were dehydrated in absolute ethanol and air-dried for a 12-h minimum at room temperature.

To observe GC- and AT-rich regions, meristematic tissue was stained respectively with chromomycin A_3 (CMA) (Serva, France) according to Schweizer (1976) and Kondo and Hisume (1982), and with Hoechst 33258 (Ho; Sigma) according to Martin and Hesemann (1988) with minor modifications.

An epifluorescence Zeiss Axiophot microscope with filter set 01 (excitation 365, emission 480 nm long pass) for Ho staining and filter set 07 (excitation 457 emission 530 nm long pass) for CMA staining was used for the cytological investigation. Images were captured with a Princeton Micromax camera.

For numerical karyotype analysis, chromosomes from nine metaphase plates were measured for *C. brevifolia*, and five for each of *C. atlantica*, *C. deodara* and *C. libani*. Chromosomes were identified and ordered according to their total length, arm ratio and fluorochrome banding patterns.

Ideograms were drawn from mean values, and chromosome types were determined according to Levan et al. (1964) as modified by Schlarbaum and Tsuchiya (1984). Karyological features were evaluated as total length (TL), the ratio (r) between the long and short arms, the relative length (RL) of each chromosome (100× total length of each chromosome/∑TL), the global asymmetric index (ASI) where ASI%=(∑ long arms/∑ total length of all chromosomes ×100), and the ratio (R) between the longest and the shortest chromosome pair. The position of a GC-rich band on a chromosome arm was recorded as the distance from the centromere relative to the total arm length of the corresponding arm.

Results

Flow cytometry

The best results in terms of nuclear quality and stability were obtained with our modified Galbraith buffer. The addition of 1% PEG 8000 neutralised phenolic and tannin compounds. Trials using *Cedrus* embryos and leaf nuclei stained with mithramycin showed poorly defined peaks with slow degradation giving large coefficients of variation. A marked improvement was obtained upon adding a low level of formaldehyde which is known to fix tannins and proteins during the extraction of nuclei (Teoh and Rees 1976). These improvements of the extraction buffer allowed a quality analysis with a highly reproducible fluorescence index ($2C_{Cedrus}/2C_{Pisum}$). Analysis of *Cedrus* tissues then yielded DNA histograms with coefficients of variation in the 2 C peaks generally from 2 to 4%. *P. sativum* was used as an internal standard since its 2 C and 4 C peaks did not overlap with cedar nuclei, in contrast to our initial choice, hexaploid wheat.

For ploidy level comparisons, all *Cedrus* embryos tested produced one peak, having almost the double intensity of the fluorescence peak of the corresponding endosperm. The mean ratio (2n/n) obtained was 1.92. Furthermore, all other vegetative tissues that we analysed, needles, stems and roots, were shown to be diploid.

Table 2 summarises the flow cytometric data, the nuclear DNA content, and the GC and the AT%, obtained using nuclei isolated from the 11 *Cedrus* populations. The $2C_{Cedrus}/2C_{Pisum}$ fluorescence index was highly reproducible within populations, as shown by the relative SD expressed as a coefficient of variation for a genome-size estimation less than 2%.

A notable feature of the *Cedrus* complex is that the five populations of *C. libani* and the four populations of *C. atlantica* contained uniform DNA values irrespective of their geographical origin. The mean DNA values (2 C) for *C. atlantica*, *C. brevifolia*, *C. deodara* and *C. libani* were respectively 32.4±0.20 pg, 31.8±0.64 pg, 32.6±0.40 and 32.5±0.41 pg. Within and among populations of the four species, differences in DNA content were small and not statistically significant. Nevertheless, some artificially high values up to 41 pg were observed in the Bcharre population with propidium iodide stain-

Table 3 Morphometric data for the chromosomes of the four *Cedrus* species. L, long arm; S, short arm; Relative length (RL), 100 x chromosome length/∑Total length; Arm ratio (r), ratio between the long and short arms; SD, standard error. Position of a GC rich band on a chromosome arm was recorded as the distance from the centromere relative to the total length of the corresponding arm. On the basis of centromere position, chromosomes were divided into two types: Types A for M, median point (r=1), and m, median region ($1<r<1.3$); Type B for m-sm, median-submedian region ($1.3<r<1.7$), and sm, submedian region ($1.7<r<3$)

Chromosome pairs		S (µm)	L (µm)	Total length ±SD (µm)	Arm ratio r	Relative length RL	Position of CMA intercalary band	Chromosome type
C. atlantica								
Type A	I	6.5	7.3	13.8 ± 0.86	1.12	9.92		m
	II	6.3	6.9	13.2 ± 0.81	1.09	9.50		m
	III	6.0	7.0	13.0 ± 0.69	1.16	9.35	III L 4.4	m
	IV	6.0	6.4	12.4 ± 0.89	1.06	8.92		m
	V	5.7	6.5	12.2 ± 0.74	1.13	8.78		m
	VII	5.8	6.2	12.0 ± 0.63	1.07	8.63		m
	VI	5.8	6.1	11.9 ± 1.03	1.04	8.56	VII S 3.7	m
	VIII	5.4	5.9	11.3 ± 0.32	1.08	8.13		m
	IX	4.7	6.1	10.8 ± 0.62	1.30	7.77		m
Type B	X	3.9	6.2	10.1 ± 0.90	1.59	7.27		m-sm
	XI	3.6	5.8	9.4 ± 0.56	1.60	6.76		m-sm
	XII	3.6	5.3	8.9 ± 0.79	1.46	6.40		m-sm
C. brevifolia								
Type A	I	6.3	7.0	13.3 ± 1.57	1.10	9.82	I S 3.8	m
	II	6.1	6.5	12.6 ± 1.72	1.06	9.30		m
	III	6.0	6.4	12.4 ± 1.66	1.06	9.15		m
	IV	6.0	6.3	12.3 ± 1.54	1.05	9.08		m
	V	5.8	6.2	12.0 ± 1.57	1.06	8.86		m
	VI	5.6	6.2	11.8 ± 1.53	1.11	8.71	VI S 3.3	m
	VII	5.3	6.1	11.4 ± 1.44	1.14	8.41		m
	VIII	4.8	6.1	10.9 ± 1.35	1.26	8.04	VIII S 2.8	m
	IX	4.6	5.9	10.5 ± 1.26	1.27	7.75		m
Type B	X	3.8	6.2	10.0 ± 1.19	1.62	7.38		m-sm
	XI	3.7	5.8	9.5 ± 1.23	1.56	7.01		m-sm
	XII	3.8	5.5	8.8 ± 0.96	1.66	6.49		m-sm
C. deodara								
Type A	I	5.3	6.8	12.1±0.06	1.28	9.01	I S 3	m
	II	5.9	5.9	11.8±0.16	1.0	9.28	II L 2.9	M
	III	5.6	6.0	11.6±0.03	1.07	9.12	III S 3.2	m
	IV	5.5	5.9	11.4±0.01	1.07	8.96		m
	V	5.3	5.7	11.00.01	1.05	8.65		m
	VI	5.2	5.6	10.8±0.01	1.07	8.48		m
	VII	5.4	5.4	10.8±0.02	1.00	8.48		M
	VIII	5.0	5.2	10.2±0.09	1.04	8.02		m
Type B	IX	3.9	6.3	10.2±0.01	1.61	8.02		m-sm
	X	3.4	6.0	9.4±0.04	1.75	7.39		sm
	XI	3.3	5.8	9.1±0.02	1.75	7.15		sm
	XII	3.3	5.5	8.8±0.10	1.66	6.92		m-sm
C. libani								
Type A	I	6.7	6.9	13.6±0.31	1.03	9.76	I S 4.4	m
	II	6.0	7.0	13.0±0.36	1.16	9.33		m
	III	6.0	6.7	12.7±0.46	1.11	9.12		m
	IV	6.0	6.5	12.5±0.29	1.08	8.97		m
	V	6.0	6.4	12.4±0.41	1.06	8.90	V S 4	m
	VI	5.6	6.6	12.2±0.49	1.17	8.76	VI S 3.8	m
	VII	5.7	6.3	12.0±0.48	1.10	8.61		m
	VIII	5.1	6.0	11.1±0.21	1.17	7.97		m
Type B	IX	4.4	6.3	10.7±0.29	1.43	7.68		m-sm
	X	4.1	6.2	10.3±0.52	1.51	7.40		m-sm
	XI	3.8	6.0	9.7±0.42	1.56	6.96		m-sm
	XII	3.5	5.5	9.1±0.47	1.56	6.53		m-sm

ing, prior to our modification of buffers. Theses early and unreproducible values were discarded. Indeed, the laborious AT and GC analyses subsequently gave further support to our conclusion that the genomes among the four species were similar.

Additionally, the AT content was estimated with Hoechst as 59.1±0.21% and the GC content as 40.4± 0.26%. The sum of the AT and GC% added up to 99.5%, which gives confidence to the data. Correcting AT+GC to 100%, the mean of these two estimates may be ex-

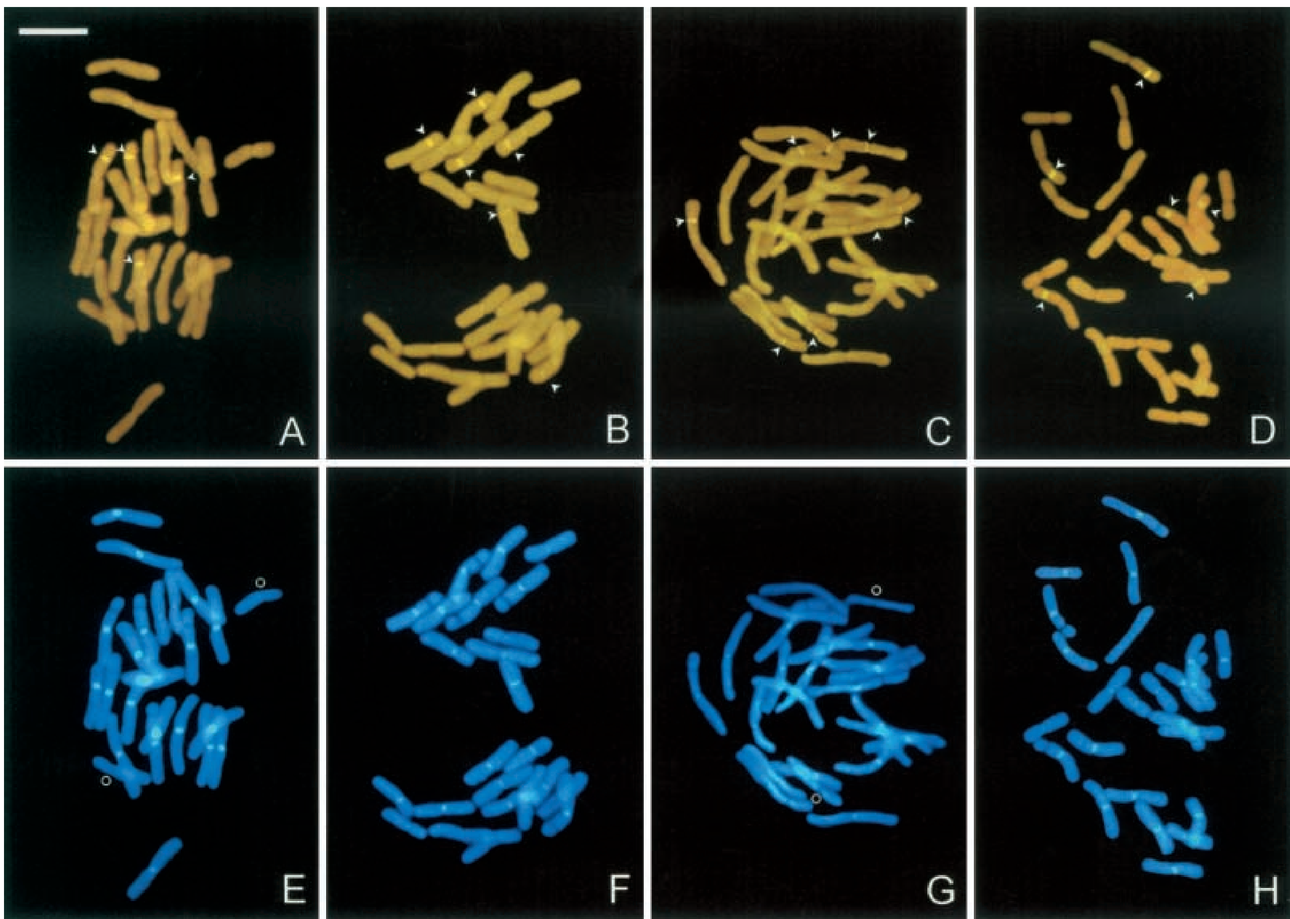

Fig. 1A–H Metaphase plates of the four *Cedrus* species. Diploid chromosome complement of *C. atlantica* (**A**, **E**), *C. brevifolia* (**B**, **F**), *C. deodara* (**C**, **G**) and *C. libani* (**D**, **H**) stained with chromomycin and Hoechst respectively. *White circle* indicates absence of Ho staining at the centromeric region. *Arrowhead* indicates CMA-rich sequences. Bar=10 µm

pressed as 40.7% GC. No significant difference was observed between the embryonic and other tissues sampled (data not shown).

Karyotype analysis

In all metaphase plates analysed in root tips in the seedlings of *Cedrus*, we observed a diploid chromosome number of 2n=2x=24. Morphometric data concerning metaphase karyotypes are presented in Table 3. The karyotypes of the four species were similar in size and symmetry. Chromosome length varied from 8.8 to 13.8 µm. Total chromosome length and the asymmetric index were respectively 139 µm and 54.5 for *C. atlantica*, 135.5 µm and 54.8 for *C. brevifolia*, 127.2 µm and 55.0 for *C. deodara* and 139.3 µm and 54.7 for *C. libani*. The chromosome complement was composed of eight to nine similarly large metacentric chromosome pairs (Type A) and three to four smaller submetacentric chromosome pairs (Type B). The long metacentric chromosomes were so similar in shape and length that they were hardly distinguishable, notably for neighbouring chromosomes from I to VII. Therefore, the identification of neighbouring chromosomes carried a relatively high probability of inverted order. For example, ordering chromosome pairs III to IV and V to VII in *C. libani* as well as the designation of the short and long arms of pairs I to VIII were all considered tentative and might change if less-condensed chromosome metaphases are studied. Only the last two or three pairs could be easily identified according to their total length (TL) and centromere position.

Fluorochrome banding

Fluorochromes revealed bands on particular areas of cedar chromosomes. The guanine-specific fluorochrome CMA showed large fluorescent bands at intercalary regions. Intercalating bands probably correspond to secondary constrictions and nucleolar organising regions (NORs). After imaging and de-staining CMA, the slides were stained with the AT-specific fluorochrome Ho. Ho bands appeared in nearly all paracentromeric chromosome regions. Conversely, all those regions that had stained intensely with CMA appeared negative after Ho staining, confirming that they were essentially GC-rich regions.

Table 4 Karyotype characteristics of the four *Cedrus* species after fluorochrome staining

Cedrus species	Chromosome number (2n)	CMA-positive bands	Ho-positive bands	Ho-negative bands
C. atlantica	24	4	22	4
C. brevifolia	24	6	24	6
C. deodara	24	8	22	8
C. libani	24	6	24	6

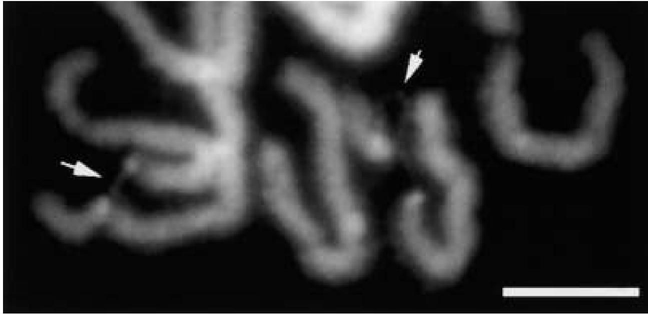

Fig. 2 Chromosomes of *C. libani* at the end of prophase. The *white arrows* show CMA staining on both sides of the secondary constrictions. Bar=4 µm

Four intercalary CMA bands were distinguished in *C. atlantica* (Fig. 1A) and 22 Ho bands were located in the paracentromeric regions of all chromosomes except for the smallest chromosome pair (Fig. 1E). For *C. brevifolia* and *C. libani*, CMA and Ho patterns were similar: six intercalary CMA bands (Fig. 1B and D) and 24 Ho bands (Fig. 1F and H) in the centromeric regions were detected. However, in *C. brevifolia* one CMA band was less intense than the five others. For *C. deodara*, we found eight CMA bands with six large intercalary bands located in the secondary constriction regions and two thin bands ahead of the centromeric region in the smallest chromosome pair (Fig. 1C). Ho staining displayed bands in all paracentromeric regions except for the shortest chromosome pair (Fig. 1G).

In some mitoses at late prophase, break points were localised in the chromosomes at the level of intercalary CMA bands (Fig. 2). Moreover, similar-sized CMA bands were observed on both sides of the break point joined together by CMA-stained chromatin.

The position of CMA and Ho bands is presented on the ideograms shown in Fig. 3. The signal strengths were usually strong. With CMA staining, minute hot spots were detected in some metaphase plates of *C. atlantica*, *C. brevifolia* and *C. libani*, located in the centromeric region of one of the chromosome pairs having the intercalary CMA band and in the centromeric region of the shortest pair. Nevertheless, we have not reported these spots in the ideogram because their presence was not always reproducible. Table 4 summarizes the fluorochrome banding pattern of the four *Cedrus* species.

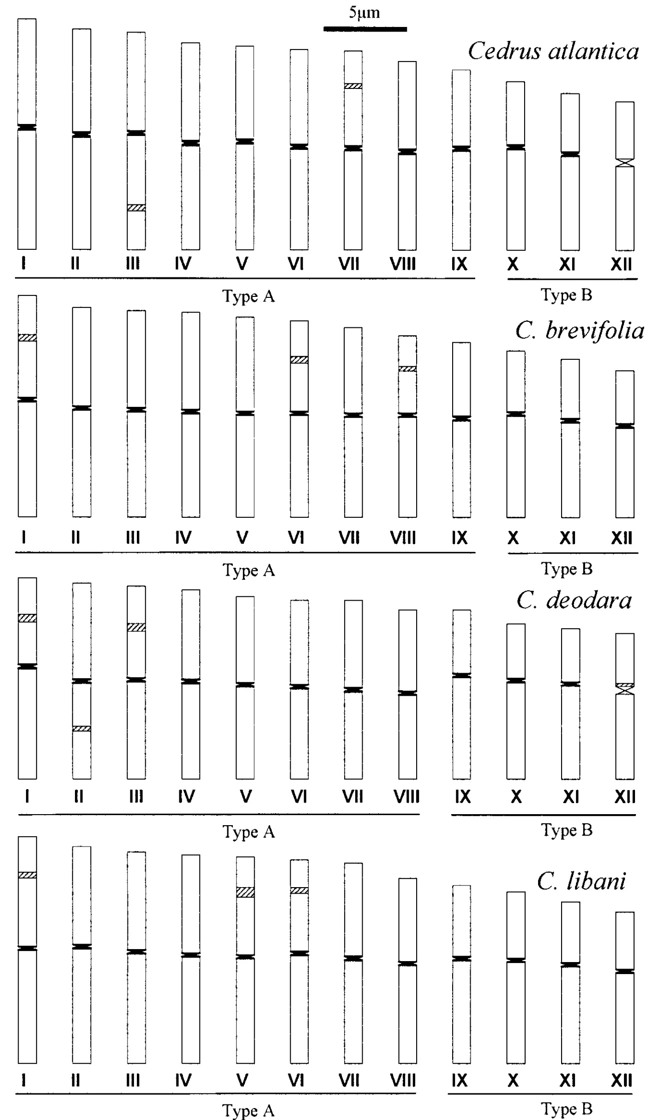

Fig. 3 Ideograms of the four *Cedrus* species. *Solid regions* represent AT-rich sequences stained by Hoechst; *striped bars* represent GC-rich sequences stained by chromomycin. Bar=5 µm

Discussion

The *Pinaceae* apparently have higher and more-dispersed DNA levels than other families of gymnosperms (Ohri and Khoshoo 1986). Although numerous studies have addressed intra- and inter-specific variability of nuclear DNA content in the *Pinaceae* (Ohri and Khoshoo

1986; Wakamiya et al. 1993; Valkonen et al. 1994; Kohler et al. 1996; O'Brien et al. 1996; Murray 1998), no information was available on the C values of cedar. Presenting many advantages over Feulgen microdensitometry, laser flow cytometry is an accurate, reliable and easy method to assess genome size and GC% in plants (Michaelson et al. 1991; Marie and Brown 1993; Kohler et al. 1996). The flow cytometric procedure used in this study showed a constancy of DNA content among the four *Cedrus* species. In *Cedrus*, these 2 C DNA values, 32.5 pg/2 C (or 15.7×10^9 base pairs per 1 C), are modest compared to the large genome sizes observed in some gymnosperms and especially in certain *Pinus* species. For example, in *P. pinaster* Ait. and *P. Lambertiana* Dougl., values of 51.4 pg/2 C (data not shown) and 63.5 pg/2 C (Wakamiya et al. 1993; Murray 1998) were respectively found. *Cedrus* has a genome size similar to several *Abies* species; *A. sibirica* Ledeb. with 31.6 pg/2 C (Ohri and Khoshoo 1986) and *A. alba* Mill. with 33.1 pg/2 C (Murray 1998). *C. libani* and *C. atlantica* showed no significant variation of DNA amount even between populations 2000 km distant from one from another (Table 1). This contrasts with North American *Pinaceae* species for which intraspecific variations of DNA content have been reported (Miksche 1968). However, reliable estimates in *Pinaceae* species may be affected by experimental artifacts, e.g. tanning (*sensu* Greilhuber 1986). Discrepancies appearing among authors for nuclear DNA amounts in the same species also could be attributed to the use of different techniques of measurement, different types of plant tissue and/or inadequate standards (Ohri and Khoshoo 1986; Wakamiya et al. 1993). Moreover, some authors obtained different 2 C DNA values within a plant according to the different tissues tested (Valkonen et al., 1994). Such differences must be handled with circumspection because of the endogenous compounds that may interfere with staining (Valkonen et al. 1994). In our investigations, no significant variation was found between measurements made from stems, needles, roots or embryos. Embryos extracted from seeds were principally used to assess genome size, and GC% estimations based upon the haploid megagametophyte tissue gave similar results, consistent with Pichot and El Maataoui (1997). Moreover, the four cedar species exhibited a uniform haploid endosperm, which may be useful for molecular investigations. *Cedrus* DNA-content constancy contrasts with other *Pinaceae* species where large interspecific differences have been found in genera such as *Picea* Dietrich, *Larix* Mill. and *Pinus* Lindl. For example, Ohri and Khoshoo (1986) reported a 1.73–fold variation in 20 *Pinus* species from North America, Europe and Eurasia. Wakamiya et al. (1993) also observed a 1.5-fold variation among 18 North American *Pinus* species. These interspecific DNA variations may be explained by possible adaptation to environmental conditions, especially to a xeric habitat (Ohri and Khoshoo 1986; Wakamiya et al. 1993). However, this hypothesis may not apply to *Cedrus* since these upland species face various environmental factors, especially temperature and water availability, depending on the growth area. Further studies are needed to clarify the importance of DNA size variation in certain Gymnosperm species and its possible adaptive significance.

To our knowledge, there is no published result regarding base composition in the *Pinaceae*. We investigated genome composition in *Cedrus* with two fluorochromes. The fact that the separate estimates for GC% and AT% added up to 99.5% (theoretically 100%) reinforces the reliability of our results. The mean estimates for these four *Cedrus* species was 40.7% GC, which is characteristic of many higher plants (Cerbah et al. 1995; Zoldos et al. 1998). At a cytogenetical level, *Pinaceae* chromosomes are long, with sizes ranging from 6 µm to 16 µm. Diploid numbers, 2n=24, are constant within the genus (Sax and Sax 1933; Saylor 1972). Karyotypes of species within genera can also show remarkably uniform structure despite differences in nuclear volume, chromosome size and DNA content (Sax and Sax 1933; Pederick 1970; Saylor 1972; Hizume et al. 1983). In their study conducted on the *Taxodiaceae* Warm., Schlarbaum and Tsuchiya (1984) modified the nomenclature for the centromere position of Levan et al. (1964) by subdividing previous classes. A new median-submedian (m-sm) class was created to provide a finer distinction between median and submedian chromosome types since differences in the centromeric position between coniferous chromosomes did not vary significantly. In our study we have adopted this nomenclature for *Cedrus* karyotyping. The analytical pairing of homologous chromosomes was possible despite the minor differences between chromosomes in total length, their differential chromosome condensation and the random distortion introduced through squashing.

In the present study, chromosomes from the four species were stained by fluorescent-banding methods to examine whether or not the fluorescent pattern was useful for the identification of certain individual chromosome pairs, to distinguish each species and to analyse intraspecific variation of banding patterns in the same population. A limitation when dealing with *Pinaceae* chromosomes is that often the arm lengths are nearly identical and thus inversion with respect to long and short arms can easily occur. Thus the assignment of GC-rich bands to one arm, i.e. short or long, is prone to error. In comparison with other *Pinaceae*, *Cedrus* contains relatively few GC-rich sequences. For example, 26 CMA bands were present in *Pinus elliottii* Engelm. (Doudrick et al. 1995), 28 in *Pinus Densiflora* Sieb. & Zucc., 23 in *Pinus Thunbergii* Parl. (Hizume et al. 1989) and 16 CMA bands were distinguished in the *Picea omorika* Purkyne karyotype and 14 CMA bands in *P. abies* (L.) Karsten (Papes et al. 1997), while only eight GC-rich bands were observed in *C. deodara*, six in both *C. libani* and *C. brevifolia*, and four in *C. atlantica*.

The presence of CMA and Ho bands is usually indicative of highly repetitive DNA sequences which appear as heterochromatin in chromosomes. In *Cedrus*, CMA-

stained regions represent less than 2% of the total genome. Our results show that the large genome size of *Cedrus*, relative to most angiosperm families, is not simply a result of the amplification of GC- or AT-rich satellite DNA families. We also hypothesise that differences in the number of CMA bands could explain the differences in DNA content between cedar and other *Pinaceae*.

Secondary constrictions were evident by phase-contrast of non-coloured metaphase plates as gaps of various widths in intercalary regions on arms. In *C. atlantica*, *C. brevifolia* and *C. libani*, two pairs of chromosomes with prominent secondary constrictions could always be identified. However, depending on the level of chromosome condensation, one other pair of secondary constriction sites with a very thin gap could be seen in *C. brevifolia* and *C. libani*. The presence of CMA bands on both sides of the break point in mitoses at the late prophase stage (Fig. 3) led us to co-locate intercalary CMA bands with secondary constriction sites known to be vulnerable regions for breakage (Saylor 1961). Analysis of ribosomal DNA distribution by in situ hybridisation, and NOR activity by $AgNO_3$ banding, may help to clarify this point. The CMA and Ho banding patterns were specific to each species. Although the assignment of GC bands to one chromosome and to the short or long chromosome arm is difficult, this study provides a cytological means to identify species. Comparative patterns of fluorochrome banding may be useful not only in identifying homologous chromosomes but also for revealing phylogenetic relationships among species (Hizume et al. 1990).

Some publications such as the Flora of Turkey (Davis 1965) and the Med-Checklist (Greuter et al. 1984) described all the Mediterranean cedars as a single species. Some authors subdivide the genus into three species, *C. atlantica*, *C. deodara* and *C. libani*, considering *C. brevifolia* as a variety of *C. libani* (Tutin et al. 1964). Given the similar banding profiles of respectively CMA and Ho obtained in *C. brevifolia* and *C. libani*, we tend to agree with the second hypothesis. Using either the amplified fragment length polymorphism (Vos et al. 1995) technique (data not shown) or isozymes (B. Fady, personal communication), many common molecular markers were observed in these two species, reinforcing the notion of a close relationship between *C. libani* and *C. brevifolia*. In contrast, the different fluorochrome banding patterns observed in *C. atlantica* and *C. libani* provides potentially useful interspecific markers and justifies their separation into two distinct species.

Little is known about the structure and composition of gymnosperm genomes. Gymnosperm plants stand to benefit from the resolution of in situ hybridisation analysis since karyotypes are remarkably conserved among gymnosperms in comparison to angiosperms (Sax and Sax 1933; Saylor 1972). We plan to study ribosomal DNA distribution systematically in the four species by in situ hybridisation, and NOR activity by $AgNO_3$ banding in order to gain a better understanding of the phylogenetic relationships among the four species and of the karyotype evolutionary mechanisms that have operated in *Cedrus*. This may be useful for the analysis of genetic synteny and for map construction in gymnosperms.

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