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Cytological and molecular relationships between *Larix decidua, L. leptolepis* and *Larix* × *eurolepis*: identification of species-specific chromosoms and synchronization of mitotic cells

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Abstract The effects of different concentrations of hydroxyurea (HU) and aphidicolin (APH) on the mitotic index (MI) were compared in cells of embryogenic cultures of *Larix decidua, L. leptolepis,* and *L. decidua* × *L. leptolepis* (*Larix* × *eurolepis*). The highest enhancement of the MI was obtained with HU at 1.25 mM and 0.6% colchicine. In general the MI decreased with an increase of HU or APH concentration (over 1.25 mM for HU and 5 μM for APH). Detailed karyotype analyses were made on the somatic complement of *L. decidua, L. leptolepis,* and their hybrid. These karyotypes were asymmetrical and advanced, with the smaller chromosomes being more submedian than the larger ones. The topography of chromosome 7 of *L. decidua* and chromosome 9 of *L. leptolepis* was found to be the most significant cytotaxonomic characteristic in differentiating these two species. Cytological data indicate that Japanese larch (*L. leptolepis*) is phylogenetically closer to European larch (*L. decidua*) than the Siberian larch group (*L. sibirica* and *L. sukaczewii*). Chromosomes with unusually long kinetochores were found in both species and the hybrid. Hyperploid cells ($2n = 25$) were observed in the hybrid (*Larix* × *eurolepis*) material analyzed. A genomic *L. decidua* probe hybridized strongly to dots of DNA from *L. leptolepis* indicating that there is high sequence homology between these two species.

Key words Larch · Hybrid · Karyotypes · Mitotic index · DNA homology

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Introduction

Morphological characters are commonly used for species differentiation in the Pinaceae, to which both *Larix decidua* (Mill.) and *L. leptolepis* (Sieb and Zucc.) Gord. belong. In many cases, however, such characteristics are of little value because they are not distinct. Under the circumstances, karyotyping and DNA characteristics have been widely used to accurately establish phylogenetic relationships among the Pinaceae (Ehrendorfer 1976; Schlarbaum and Tsuchiya 1984; Baum et al. 1987; Seberg 1989; Orgaard and Helsop-Harrison 1994).

On the basis of previous cytological analyses, the Euro-asiatic larches can be divided into two karyotype groups: the European larches and the Siberian larches. The European larch (*L. decidua* Mill.) is found in the Alps, Sudetic Mountains, Slovakia, Poland, and Romania. The Siberian larch group includes *L. sibirica* and *L. sukaczewii.* The karyotypes of European larch and the Siberian larches are basically similar; they are composed of six pairs of long chromosomes with median to near-median kinetochores and six pairs of short chromosomes with submedian kinetochores (Sax and Sax 1933; Simak 1962, 1964). The different species can, however, be distinguished by the number of secondary constrictions. These early studies were carried out using root tips where the percentage of cells showing secondary constrictions was less than 20 and the mitotic index (MI) was relatively low (Simak 1964). This may have reduced the accuracy of karyotype comparison.

On the othe hand, the karyotype of *L. leptolepis*, a species from Hondo Island, Japan, has not been studied in detail. Japanese larch hybridizes easily with European larch in nature and artificial hybrids have been also produced in seed orchards, which suggests a close phylogenetic relationship (Hacher and Bergmann 1991). The resultant hybrid larch (*Larix* × *eurolepis* A. Henry; seeds collected from *L. decidua*) has been cited for its positive heterotic effect (Paques 1989). Its su-

periority with regard to morphological and phenological characteristics, growth traits, wood properties, and physiological parameters, has also been demonstrated (Paques 1989). However, its cytological stability has not been determined.

The aim of the present study was to (1) enhance the mitotic indices in larch cells derived from embryogenic cultures, (2) establish detailed karyotypes of *L. leptolepis* and to compare these with karyotypes of *L. decidua* using embryogenic cultures and root tips, (3) determine the mitotic stability of *Larix* × *eurolepis*, and (4) determine the degree of genomic DNA homology between *L. decidua* and *L. leptolepis.*

Materials and methods

Plant materials

Two embryogenic culture lines of *L. decidua* (L10 and L2T) and two of *L leptolepis* (L7 and L2) were used to compare karyotypes, chromosomal mitotic behaviour, and DNA similarity. Two lines of *Larix* × *eurolepis* (L5 and L8) were also analyzed. Hybrid seeds were obtained by controlled pollination using a European larch as the maternal tree and a Japanese larch tree as the pollen source. The F_1-derived seeds were collected to establish embryogenic cultures. These cultures were initiated and maintained as previously described (von Aderkas et al. 1990).

Seeds of two *L. decidua* lots, one *L. leptolepis* lot and one *Larix* × *eurolepis* lot were germinated to compare the chromosome morphology of root-tip cells with cells from cultures. These seeds were obtained from the National Seed Centre, Petawawa National Forestry Institute, Chalk River, Ontario. The seeds were placed in clear polycarbonated "Petawawa germination boxes" containing wet "Kimpak" cellulose paper and kept in a germinator at 30 °C for 12 h under fluorescent lights and 8 h at 20 °C in darkness. Seedlings with roots 2–4 cm long were collected for colchicine pretreatment.

Pretreatment

Embryonal masses were incubated in liquid MSG media (Becwar et al. 1990; von Aderkas et al. 1990), which contained different concentrations of hydroxyurea (HU) (Sigma) or aphidicolin (APH) (Sigma), on a shaker at 120 rpm for 18 h. HU and APH are DNA-synthesis inhibitors that block the cell cycle in S-phase. Hydroxyurea and APH were applied to suspended cultures as freshly prepared solutions. Three concentrations of HU and APH were tested. These included 1.25 mM, 5 mM, and 10 mM for HU and 10 μM, 15 μM, and 30 μM for APH. Concentrations of 1.25 mM HU and 15 μM APH have been found effective in cell-cycle synchronization in other plant systems (Matthews 1993; Ramulu et al. 1993; Schubert et al. 1993; Nkongolo and Klimaszewska 1994). Following incubation, the embryogenic cultures were rinsed three times with fresh liquid MSG medium without HU or APH. The cultures were then incubated in the last rinse for 6 h prior to addition of 0.06% or 0.6% colchicine. The colchicine treatment lasted 18 h. Subsequently, the cultures were spun and the supernatant removed. The cells were fixed in 3:1 ethanol: acetic acid, and hydrolysed in 1N HCL at room temperature for 1 h. The squash preparations were made in acetocarmine and examined. Control material was treated in the same way except for the omission of HU and APH.

Cytological analyses

Prophase, metaphase, and anaphase cells were analyzed in all the preparations derived from embryogenic cultures and seedling root tips so as to compare chromosomal behaviour. Chromosomes were counted in 100 metaphase and pro-metaphase cells for each treatment. The mitotic index was calculated as the percentage of cells at prophase, metaphase, and anaphase. These data were transformed by arcsin $\sqrt{\text{percentage}}$ to improve their normality and the transformed data analyzed as a completely randomized experiment, with each experimental unit containing five slides. An analysis of variance and the LSD test were computed using MSTAT-C statistical programs (MSTAT Development Team 1988).

Karyotype analyses were from several representative cells for each treatment in which the chromosomes were well separated and the secondary constrictions visible. Cells with the desirable chromosome conditions were photographed at × 100 under oil using an Olympus microscope.

The chromosomes of the photographed cells were numbered from 1 (the longest) to 12 (the shortest). To measure the chromosomes, a fine thread was laid over each chromosome in the photograph, marked, and then measured in millimeters. All measurements were conducted twice. Homologous pairs were arranged by comparing centromere positions and the presence of secondary constrictions. Averages of the paired chromosomes were taken for total length, relative length, index, and ratio. A relative value was used as the standard for chromosome lengths. The relative length of a given chromosome was determined by dividing the absolute chromosome length and the sum of all the chromosome lengths and was expressed as a percentage of the total (Tjio and Hagberg 1951). The ratio of the long arm to the short arm was used to identify centromere position for each chromosome. The nomenclature system of Levan et al. (1964) was used to determine the chromosome types as median, submedian, or subterminal. Diagrammatic presentations for the chromosomes from embryogenic cultures and seedling root tips were developed according to arm ratios and relative lengths. The idiograms representing *L. decidua* and *L. leptolepis* were based on averages for relative lengths and arm ratios. Karyotypes established from root-tip cells were compared to those of embryogenic culture cells.

Genomic DNA isolation

Total genomic DNA was obtained from *L. decidua*, *L. leptolepis*, and *Larix* × *eurolepis* cultures according to the method of J. C. Leggo and R. Rutledge (Petawawa National Forestry Institute, personal communication). Genomic DNA was isolated by grinding embryonal masses in liquid nitrogen with 3 ml of extraction buffer (50 mM Tris-HCL, 50 mM EDTA, 50 mM NaCl, 1% Na N-laurylsarcosine) per gram fresh weight. Cellular debris was removed by low-speed centrifugation and DNA banded in CsCl. Following collection of the DNA band, the ethidium bromide was removed by extraction with isoamyl alcohol (Maniatis et al. 1982) and DNA-precipitated by the addition of an equal volume of ddH_2O followed by the addition of two volumes of absolute ethanol.

Probe preparation

A whole genomic probe was prepared using *L. decidua* DNA and the Megaprime DNA labelling system as per the instructions provided with the kit (Amersham, UK). The probe was spun through a sephadex column to remove unincorporated nucleotides. The efficiency of the preparation was determined by dotting 1 μL onto a glass filter and reading scintillation counts before and after the filter was washed with 5% TCA and 95% EtOH.

Slot blot

Samples of DNA were serially diluted and denatured with 0.5 M NaOH. Fifteen Microliters of each diluted DNA sample was dotted onto a Biotrans nylon membrane, resulting in a total amount of 500, 250, 100, 50 and 10 ng being placed within each slot, respectively. The DNA was crosslinked to the membrane using a UV crosslinker through two optimal linking cycles. The blot was prehybridized at 60 °C using 5% skimmed milk in PBS diluted 25 in standard hybridization buffer [1M NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.5), 1% Na N-laurylsarcosine]. Following 2.5-h prehybridization, hybridi-

zations were conducted overnight in hybridization buffer containing 240 ng of ^{32}P-labelled *L. decidua* genomic DNA and 1 mg of denatured salmon sperm DNA. Blots were washed in 2 × SSC + 0.1% SDS and 0.2 × SSC + 0.1% SDS at room temperature and 0.16 × SSC + 0.1% SDS at 60 °C for 15 min. Blots were then exposed to X-ray film for 24 h using an intensifying screen.

Densitometry

The X-ray film was scanned in a MSF-300ZS Flatbed Image Scanner and the digital image analyzed by a NIH Image 1.31 software package. An area of 680 pixels was measured that covered the bands. The background reading was also taken and subtracted from the values.

Results

Effect of hydroxyurea and aphidicolin on mitotic index

The mitotic index (MI) data are summarized in Table 1. Data from different lines belonging to the same species were pooled because their variances were similar. Significant enhancement of the MI was observed when the colchicine concentration was increased from 0.06% to 0.6% in cells of *Larix* × *eurolepis* embryogenic cultures. Metaphase cells doubled when the colchicine concentration was increased.

Hydroxyurea and aphidicolin were used at different concentrations to enhance the mitotic indices (MI) of cells derived from embryogenic cultures. Maximum levels of the MI were obtained after 1.25 mM hydroxyurea and 0.6% colchicine treatment, resulting in a MI ranging from 50 to 70% (Table 1). These were three-to-five-fold higher than the MI obtained in the control treatment without HU or APH (Fig. 1a, b). In the *L. decidua* and *L. leptolepis* material studied, an increase of HU concentration from 1.25 mM to 5 mM was less effective, resulting in only a 25 to 27% increase of the mitotic index (two-fold higher that the MI obtained with the control treatment). In general the APH at the concentrations used was not as effective in increasing the MI as was HU. As with HU, the MI significantly decreased with an increase in APH concentration.

Table 1 Percentages of cells at prophase, metaphase, anaphase, telophase and interphase and mitotic index (arcsin values in parentheses) in preparations from *L. decidua, L. leptolepis*, and *Larix* × *eurolepis* cell cultures pretreated with hydroxyurea or aphidicolin and colchicine

Embryogenic cultures	Pretreatment	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Interphase (%)	Mitotic index[a] (%)
L. decidua	0.6% colchicine	1.9	8.3	0.0	13.3	76.5	10.2 (18.6)
	1.25 mM HU + 0.6% colchicine	3.3	47.5	0.0	19.1	30.1	50.8 (45.5)
	5 mM HU + 0.6% colchicine	2.5	23.0	0.0	11.0	63.5	25.5 (30.3)
	5 μM APH + 0.6% colchicine	2.0	17.9	0.0	8.8	71.3	19.9 (26.5)
	15 μM APH + 0.6% colchicine	1.0	16.0	0.0	14.0	69.0	17.0 (24.4)
	30 μM APH + 0.6 colchicine	3.1	6.1	0.0	11.8	79.0	9.2 (17.7)
L. leptolepis	0.6% colchicine	2.1	11.0	0.0	16.5	70.4	13.1 (21.2)
	1.25 mM HU + 0.6% colchicine	3.2	47.7	0.3	15.0	33.8	51.2 (45.7)
	5 mM HU + 0.6% colchicine	2.7	24.1	0.0	9.0	64.2	26.8 (31.2)
	5 μM APH + 0.6% colchicine	2.5	18.3	0.0	9.0	70.2	20.8 (27.1)
	15 μM APH + 0.6% colchicine	0.7	14.7	0.0	12.2	72.5	15.4 (23.1)
	30 μM APH + 0.6% colchicine	2.6	8.5	0.0	11.0	78.9	11.1 (19.5)
Larix × *eurolepis*							
	0.06% colchicine	4.0	6.4	0.1	13.9	75.6	10.5 (18.9)
	1.25 mM HU + 0.06% colchicine	5.9	38.1	0.0	12.8	43.2	44.0 (44.6)
	0.6% colchicine	5.1	16.8	0.9	16.1	61.1	22.8 (28.5)
	1.25 mM HU + 0.6% colchicine	11.1	55.6	1.0	12.3	20.0	67.7 (55.4)
$LSD_{0.05}$		–	–	–	–	–	4.2 (11.8)
$LSD_{0.01}$		–	–	–	–	–	5.7 (13.8)

[a] Average of five replications. Angular-transformed means in parentheses

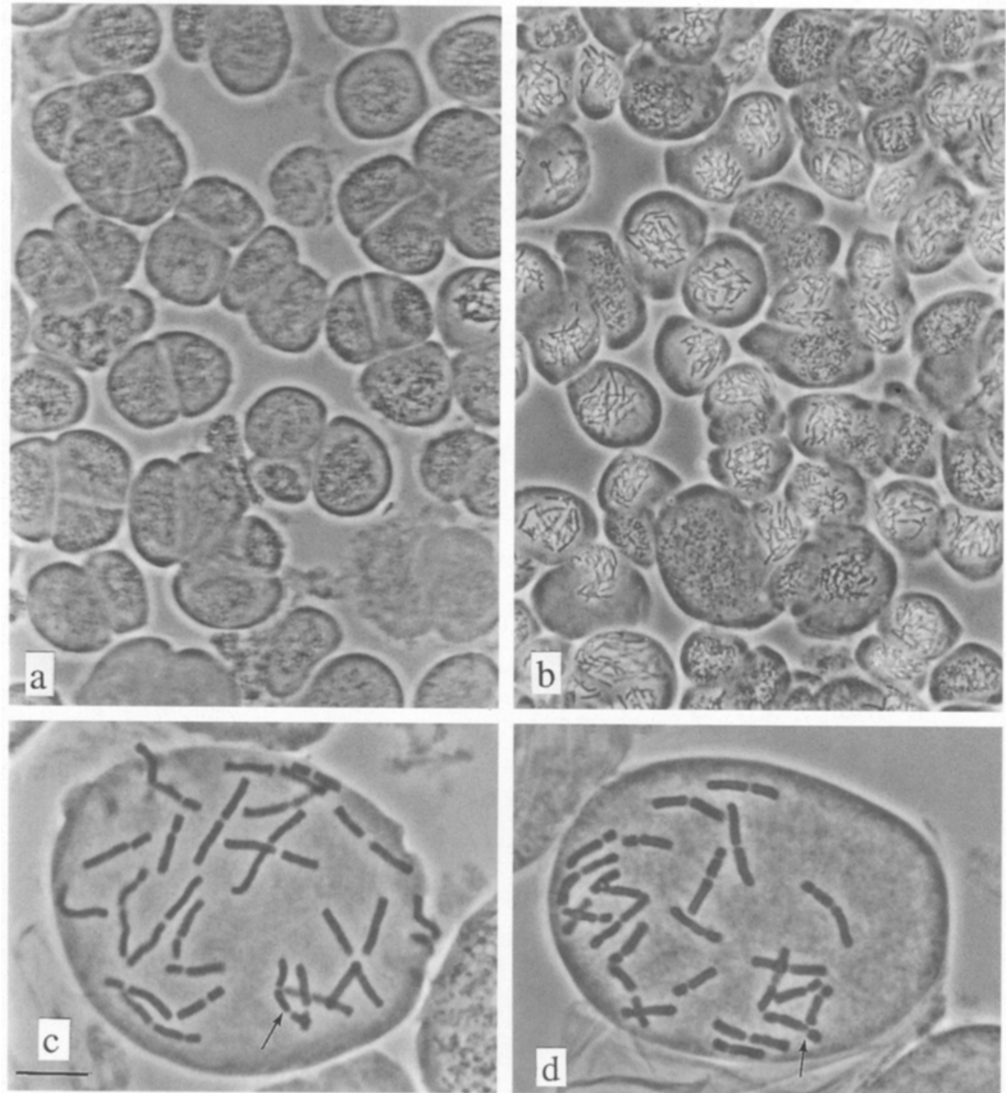

Fig. 1a–d Squash preparation of cells from *L. decidua* embryonal masses without synchronization (**a**) and after synchronization for 18 h in 1.25 m*M* Hydroxyurea (**b**); and somatic metaphase chromosomes (2n = 24) of *L. leptolepis* (**c**) and *L. decidua* (**d**). *Arrows* indicate a species-specific chromosome

Chromosome morphology

Larix decidua. The chromosome number of *L. decidua* was found to be 2n = 24 (Fig. 1d), which confirms earlier reports; for example, Simak (1964). There was not notable differences in chromosome morphology among the different lines analyzed. The five longest chromosome pairs (1, 2, 3, 4, 5) have median kinetochores (m-type). Chromosome pair 6 has a median to submedian kinetochore (msm-type) and the chromosome pairs 7, 8, 9, 10, and 11 were of the sm-type (submedian kinetochores). The smallest pair of chromosomes (chromosome 12) has a subterminal or submedian kinetochore (Figs. 2a and 3a).

A distinctive secondary constriction was observed on the short arm of chromosomes 2 and 4 and on the long arm of chromosome 7. This heterobrachial chromosome 7 was the easiest to identify and can be used as the main reference chromosome for distinguishing the *L. decidua* genome. One chromosome pair exhibited a long kinetochore region. This region was neither an artifact nor an aberration but rather a consistant feature of the chromosome complement observed in several cells.

In general, the diagrammatic representations of the chromosomes of *L. decidua* show that they are distributed in a dispersed pattern (Fig. 3a). The karyotype is relatively asymmetrical, with wide differences in chromosome size and a tendency for the smaller chromosomes to have non-metacentric kinetochores.

Larix leptolepis. The diploid chromosme number of *L. leptolepis* is 2n = 24 (Fig. 1c). Chromosome pairs 1, 2, and 5 were of the M-type (median kinetochore point). Chromosome pairs 4 and 6 were of the m-type (median kinetochore region) but 3, 9, and 12 were of the msm-type (median to submedian kinetochores). The other chromosome pairs (7, 8, 10 and 11) were of the sm-type (submedian kinetochores) (Fig. 2b). Secondary constrict-

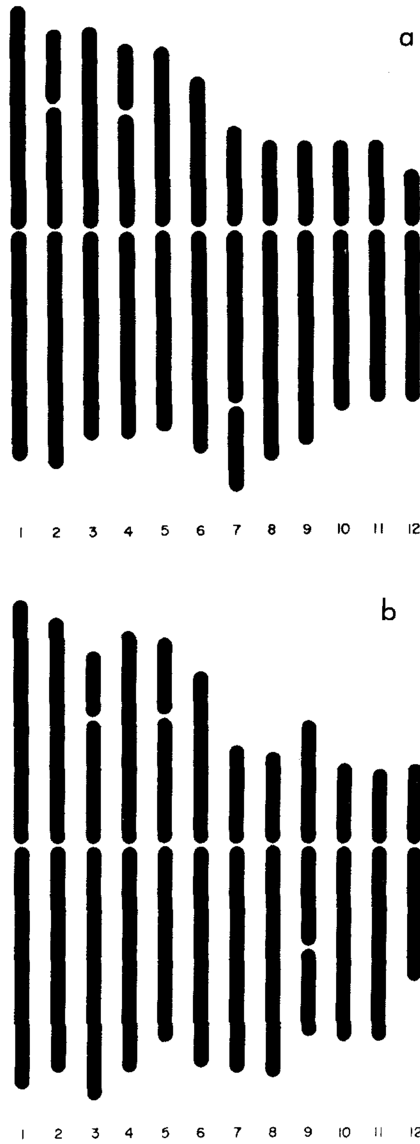

Fig. 2 Idiograms representing the chromosomes of *L. decidua* (**a**) and *L. leptolepis* (**b**). The chromosomes are numbered by decreasing order of chromosome length

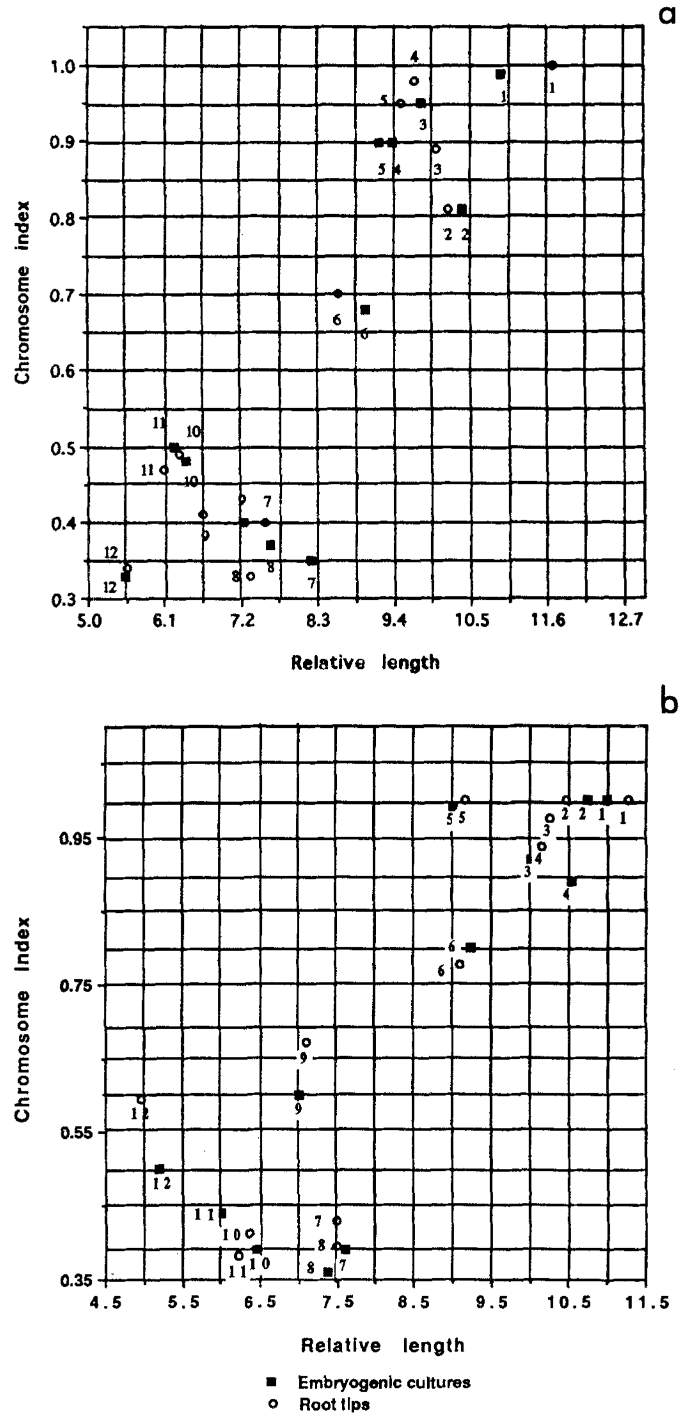

Fig. 3 Diagrammatic presentations of *L. decidua* (**a**) and *L. leptolepis* (**b**) using enbryogenic cultures and root tips. Relative lengths (X-axis) were derived by averaging the percentages of the total chromosome length for individual chromosomes. The chromosome index is the average of the short arm to long arm ratios (Y-axis)

ions were observed on the short arm of chromosomes 3 and 5 and on the long arm of chromosome 9. This heterobrachial chromosome 9 was easy to identify and can be used to distinguish *L. leptolepis* from *L. decidua*. In fact, the topography of this chromosome pair was different from that of chromosome 7 used for the identification of *L. decidua* (Fig. 3b). As in *L. decidua*, one pair of chromosomes have an unusually long kinetochore region. The diagrammatic representation indicates some variation in chromosome size, with many chromosomes having non-metacentric kinetochores (Figs. 2b and 3b). This type of karyotype is also asymmetric and advanced, as in *L. decidua*.

Larix × *eurolepis.* A high frequency (up to 70%) of hyperploid cells with $2n = 25$ chromosomes was found in the two embryogenic *Larix* × *eurolepis* lines analyzed (Fig. 4a). Chromosome 7 from *L. decidua* and chromosome 9 from *L. leptolepis* were both easy to identify due to their characteristic topography. The other chromosomes were less easily identifiable. Chromosome with unusual kinetochores were also observed (Fig. 4c). As in

L. decidua and *L. leptolepis*, the length of this kinetochore region varied in different cells at the same stage of mitosis. Some variation was observed between homologous chromosomes in the same cell. This may be partly ascribed to artifacts. Despite the high level of hyperploidy, several cells showed normal mitotic behaviour at prophase, metaphase, anaphase, and telophase. However in a few cells, chromosomes were attached by their telomeres at prometaphase (Fig. 4b).

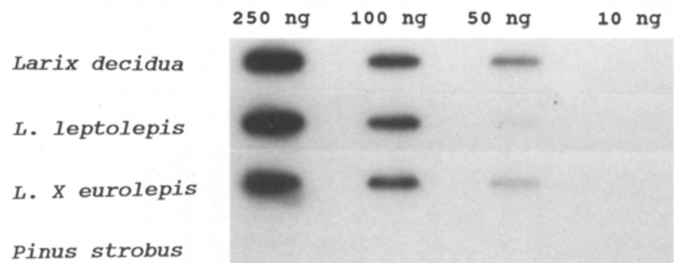

Fig. 5 Slot blots showing hybridization of a *L. decidua* genomic probe to the DNA of *L. decidua, L. leptolepis, L.* × *eurolepis* and *P. strobus*

Genomic DNA hybridization

Figure 5 and Table 2 show one of the slot blots and the hybridization intensity (DI) of DNA from *L. leptolepis, L. decidua, Larix* × *eurolepis*, and *Pinus strobus* with labelled total genomic DNA from *L. decidua*. The hybridization signal was detected as dark stains on all dots with *L. decidua, L. leptolepis*, and *Larix* × *eurolepis* while the blots of *P. strobus* DNA were not stained at all at the different concentrations used. The amount of hybridization in the slot blots shown in Fig. 6 was quantified. The relative intensities of the blots from *L. decidua, L. leptolepis* and *Larix* × *eurolepis* probed with *L. decidua* genomic DNA varied from 66% to 100%. The highest relative intensities were obtained with high DNA concentrations (250 ng and 100 ng) (Table 2).

Discussion

The highest enhancement of MI was obtained with HU at 1.25 mM and 0.6% colchicine. The fact that low levels of HU (1.25 mM) and APH (5 μM) are more effective in inducing a high MI than higher levels is contradictory to previous studies involving other plant species. Ramulu et al. (1993) found no noticeable difference in the MI of *Nicotiana plumbaginifolia* cell suspensions pretreated with HU at 10 mM and those obtained after APH pretreatment at 15 μM. Mii et al. (1987) also reported that treatment of cells with HU at 5 and 10 mM induced a higher MI than with lower concentrations (1 and 2 mM).

Overall, the maximum MI obtained with HU in this study is much higher than those reported in many previous studies. Our method yielded a MI of 20 to 40%

Fig. 4a–c Somatic metaphase cells of *Larix* × *eurolepis* showing a hyperploid cell (2n = 25) with normal chromosomes (**a**); a cell with attached chromosomes (indicated by *arowhead*) and unusual long kinetochores (indicated by *arrows*) (**b**) and highly magnified chromosomes with unusual long kinetochores (indicated by *arrows*) (**c**)

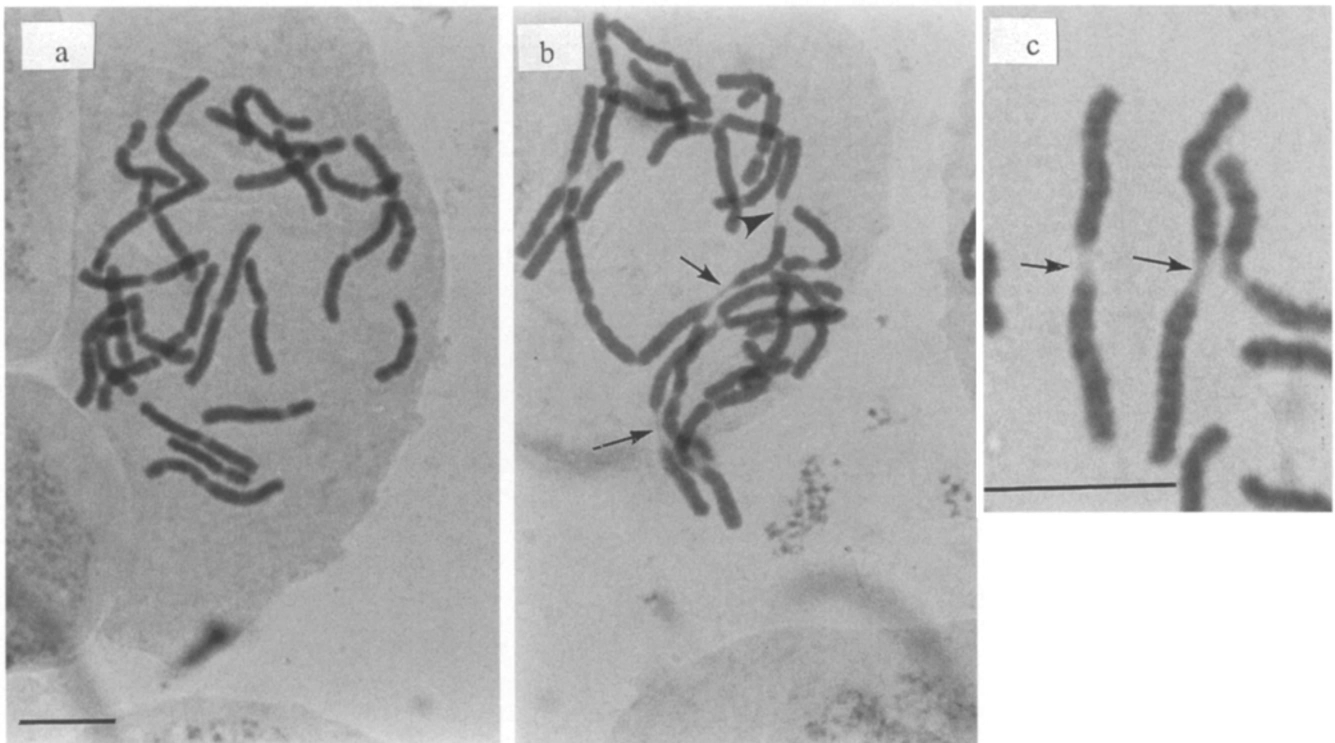

Table 2 The amounts of hybridization of a *L. decidua* genomic probe to the DNA from *L. decidua*, *L. leptolepis* and *L.* × *eurolepis* calculated relative to their hybridization to the *L. decidua* slot blot

Species	Signal relative to *L. decidua* blot (%)		
	Amount of DNA used as probe		
	250 ng	100 ng	50 ng
L. decidua	100	100	100
L. leptolepis	94	98	89
L. × *eurolepis*	100	100	66
P. strobus	5	7	8

when applied to black spruce cultures (Nkongolo and Klimaszewska 1994) and a less than 20% MI with carrot cells (Matthews 1993). Our results were, however, within the same range as those reported by Schubert et al. (1993) using the field bean *Vicia faba*. These variations are probably due to inherent differences between the plant species, growth requirements in culture and, perhaps, differences in the relative proportion of cells normally participating in cell culture growth. In fact, we found that cells from embryonal masses that were regularly subcultured had a high yield of mitotic cells and responded far better to HU and APH treatment than those that were poorly maintained.

Cytological analysis

The arm ratios and relative chromosome values did not show variation between embryogenic cultures and root tips (Fig. 3). This confirms our previous report indicating that conifer plants are cytologically stable in culture (Nkongolo and Klimaszewska 1994). Thus embryogenic cultures are suitable material for cytological characterization of tree species.

The karyotypes of both *L. decidua* and *L. leptolepis* were asymmetrical. This karyotype can be regarded as an advanced type, in which structural changes have occured, rather than a semi-advanced type as suggested by Simak (1964). Usually asymmetrical karyotypes evolve through spontaneous pericentric inversion and unequal reciprocal translocations (Stebbins 1971). The karyotypic evolution in *Larix* appears to be quite similar to that described in some Taxodiaceae by Schlarbaum and Tsuchiya (1984). Initially, unequal translocations took place between chromosomes resulting in two distinct size classes of non-metacentric chromosomes. Pericentric inversions then occurred in the large chromosomes moving the kinetochores to a more median position. Details of this type of karyotypic evolution are provided by Stebbins (1971).

The comparison of genomic diagrams and the idiograms between *L. decidua* and *L. leptolepis* indicates that, in general, the two karyotypes are similar and there is a true cytotaxonomic relationship between the two species (Figs. 3a, b). As pointed out by Schlarbaum and Tsuchiya (1984), conclusions on cytotaxonomic relationships based upon the relative proximity between paired points in genomic diagrams need to be carefully considered. Measurements of the individual chromosome arms on which such diagrams are based are subject to inaccuracy due to the quality of preparation, photographic quality, and possible unconscious bias in the measurement process. Any innate error in measurement can greatly affect the position of the plotted point. In fact, the risk of reversal of chromosome order is high within the long and short chromosome groups as the difference between chromosome length is very small. This, in part, can explain the difference in the chromosome order of *L. decidua* in Simak (1962) and the results of our study. Intraspecific variation of karyotypes is not likely to be the cause of the discrepancies between the two studies because Simak (1962) used seeds from several different provenances.

To clearly establish the cytological similarity between *L. decidua* and *L. leptolepis*, we relied upon detailed analyses of the structure and genetic architecture of the chromosomes. This analysis showed that the specific chromosomes are different. The two species each have a distinctive reference pair of heterobrachial chromosomes. This pair could be good cytological marker for species identification and the determination of phylogenetic relationships among different larch species. Chromosome 9 of *L. leptolepis* has a short arm/long arm ratio of 0.65 whereas this ratio is 0.39 for chromosome 7 of *L. decidua*. Thus the reference heterobrachial chromosomes of *L. decidua* and *L. leptolepis* are easy to distinguish from one another. The Siberian larches (*L. sibirica* and *L. sukaczewii*) that have been previously described by Simak (1964) have a secondary constriction on the short arm of two pairs of chromosome, as have *L. decidua* and *L. leptolepis*. However, they lack a secondary constriciton on any long arm. Thus the Japanese larch (*L. leptolepis*) is phylogenetically closer to the European larch (*L. decidua*) than to species in the Siberian larch group.

The presence of chromosomes with long kinetochores was unexpected. Such unusual structures have been reported only in some species of the *Taxodiaceae* (Schlarbaum et al. 1983, 1984) but not in the *Pinaceae*. Although the morphology and occurrence of these long kinetochore regions are variable, they are a very useful feature in determining phylogenetic relationships. The exact role of the long kinetochore region is unknown but similar structures in some genera of *Taxodiaceae* are thought to contain the nuclear organizer region in addition to the microtubule attachment site (Schlarbaum et al. 1983, 1984)

The superiority of *Larix* × *eurolepis* over the two parental species has been described by many tree breeders (Paques 1989). This superiority is related to growth characteristics and several other economically important traits. In our study, we showed that hybrid larch can be as easily cultured as the parent species and

that it also responded well to cytological treatments. The fact that a high frequency of hyperploid cells was found would indicate some cytological instability in derived progenies. The mechanism involved in the occurrence of these hyperploid cells was not investigated. In general cytological abnormalities are one of the factors causing partial sterility in artificially synthesized amphiploids plants. A more detailed meiotic analyses of *L. decidua* × *L. lepolepis* derivatives would be warranted to assess the degree of cytological instability.

Genomic DNA homology

Because the genomic *L. decidua* probe hybridized to dots with high and low amounts of DNA from both larch species and their hybrids it appears that there is high sequence homology between *L. decidua* and *L. leptolepis*. Moreover, the hybridization intensity (DI), a very useful diagnostic character (Dvorak and Zhang 1990), showed no differentiation between *L. decidua* and *L. leptolepis*. These data provide strong evidence that *L. decidua* and *L. leptolepis* are closely related. Thus the DNA from the two *Larix* species cannot be distinguished by the hybridization procedure. As a consequence, discrimination between these two species in interspecific hybrids using genomic DNA as a probe will be difficult and will require the use of blocking DNA and stringency control as described by Anamthawat-Jonsson et al. (1990).

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