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Mitochondrial DNA variation in somatic embryogenic cultures of *Larix*

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Abstract Southern hybridization analysis using wheat mitochondrial gene-specific probes indicates that changes in mitochondrial genomic organization and the relative representation of certain genomic regions occur during in vitro somatic embryogenic cell culture of *Larix* species. We observed differences in the mitochondrial (mt)DNA hybridization patterns between somatic embryogenic cell cultures and trees grown from seed for *Larix leptolepis, L. decidua*, and the reciprocal hybrids of these two *Larix* species. This is the first study to describe the correlation of molecular changes in a gymnosperm mitochondrial genome with in vitro somatic embryogenic cell culture. Quantitative differences in mtDNA hybridization signals were also observed among a 4-year-old somatic embryogenic cell culture of *Larix* × *eurolepis* trees regenerated from this culture, and the seed source tree from which the somatic embryogenic cell cultures were initiated.

Key words Mitochondria · *Larix* · RFLP · Somatic embryogenesis · Somaclonal variation

Introduction

Plant cell culture involves the establishment and proliferation of undifferentiated cells under in vitro conditions for many cell generations. When followed by the regeneration of plants this process can be used to produce plant clones that are phenotypically and genetically identical to the original material. However, the passage of cells through in vitro culture may also result in a wide range of variation in many plant species, a phenomenon termed somaclonal variation (Larkin and Scowcroft 1981). In vitro culture conditions appear to affect the stability of plant genomes, with various plant species and genotypes responding differently. Many kinds of genetic variation have been observed and particularly frequent are point mutations, activation of transposons, and changes in chromosome structure and number (De Klerk 1990). Somaclonal variation may provide a valuable source of genetic variants for plant breeding purposes, but it is undesirable when genetic stability is required, as in the production of cloned plantlets. Changes in ploidy levels have been associated with in vitro culture for several angiosperms (D'Amato 1990). However, most gymnosperm callus and tissue cultures show no evidence of polyploidy (Von Arnold 1982; Wochok and Abo El-Nil 1977; Partanen 1963; Mehra and Anand 1979), except in certain *Pinus* species where the genetic instability was attributed to the presence of auxin in the culture medium (Renfroe and Berlyn 1984). In particular, both adventitious shoots and shoots regenerated from callus of *Larix* × *eurolepis* Henry have been found to retain the diploid DNA content identical to that of the parent plant (Wyman *et al.* 1992).

Although the majority of these genetic changes occur in the nuclear genome, analyses of plant mitochondrial (mt) DNA (and, in a few cases, chloroplast (cp) DNA) indicate that in vitro cell culture can result in structural changes such as inversions, deletions/insertions, and transpositions in the organization of the organellar genomes. Plant mtDNA has the distinctive property of existing as a heterogeneous population of subgenomic molecules arising from recombination across repeated elements so that restriction profiles have a non-stoichiometric nature (reviewed by Andre et al. 1992). MtDNA recombination in somatic hybrids of higher plants was first suggested by Belliard et al. (1979); they found two varieties of tobacco displaying distinctly different mtDNA restriction patterns after protoplast fusion, having new restriction fragments that could not be attributed to either parent. The appearance of novel restriction fragments, the disappearance of subgenomic DNA molecules, and the quantitative variation in

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the abundance of certain restriction fragments encompassing a given recombinational repeat have been reported in studies of the mitochondrial genome of wheat tissue cultures (Morere-LePaven et al. 1992a; Hartmann et al. 1987; Rode et al. 1987).

Novel mitochondrial genomic organizations have been determined to be associated with long-term in vitro culture in rice (Chowdhury et al. 1990) and *Brassica* (Shirzadegan et al. 1991), as well as in wheat (Hartmann et al. 1989). The rearranged forms of these mitochondrial genomes were found to increase and decrease in varying proportions with continued culturing. The longer the duration of the in vitro step prior to regeneration, the higher the probability of obtaining mtDNA variability in regenerated plants. It has been proposed that the rapid structural alterations in vitro result from preferential amplification and reassortment of minor pre-existing forms of the genome rather than de novo rearrangements. Furthermore, differences in the organization of the mitochondrial genome between wheat embryogenic and non-embryogenic callus cultures have been detected, suggesting that a particular mitochondrial genome organization may be correlated with the ability of cultured wheat cells to regenerate whole plants (Morere-LePaven et al. 1992b; Rode et al. 1988). However, in certain cases, comparisons of plants and cultured cells have not revealed differences in mtDNA restriction fragment patterns (Matthews and DeBonte 1985; Kemble et al. 1988).

In this paper, we survey several regions of the mitochondrial genome of somatic embryogenic cells of *Larix leptolepis*, *L. decidua,* and the reciprocal hybrids of these two species. We used wheat mitochondrial gene-specific probes in Southern hybridization analyses, similar to those used in studies that established the maternal inheritance of *Larix* mtDNA (DeVerno et al. 1993). Comparisons were made between these hybridization patterns and those of the seed trees used to initiate the somatic embryogenic cell cultures.

Materials and methods

Plant materials

The larch trees used in this study are part of Experiment no. 252-C, a demonstration plot of larch species and hybrids located at the Petawawa Research Forest, Chalk River, Ontario, Canada. The trees were sown in 1977, transplanted in 1978, and planted at the current location in 1980 at a spacing of 5 m×5 m. Nine trees of each of the four following seedlots were planted: *Larix leptolepis* Lot J.8951–79050; *L. decidua* Lot J.7462–748524; Hybrid *L. decidua* × *L. leptolepis* Lot J.9982-748526; Hybrid *L. leptolepis* × *L. decidua* Lot J.9981-748525. The seedlot of *L. decidua* originated from Jagesborg, Denmark (improved Sudeten larch); *L. leptolepis,* from Central Honshu, Japan, Nagaro Provenance; and both open-pollinated hybrids were from Germany. The in vitro cultures included somatic embryogenic cell cultures of *Larix*×*eurolepis* hybrids, *L. decidua,* and *L. leptolepis* all of which had been maintained on modified Murashige and Skoog (1962) medium containing 1460 mg/l glutamine, 2.0 mg/l 2,4-D, and 0.5 mg/l BAP, with weekly subculturing for 4 years. Trees regenerated from 3-month-old somatic embryogenic cultures of *Larix*×*eurolepis* (L287) were established and have been maintained in a plot at the Petawawa National Forestry Institute nursery since 1989.

Plant DNA isolation and analysis

Total DNA was isolated from *Larix* somatic embryogenic cell cultures using a method adapted from Mettler (1987). A modification of the procedure of Murray and Thompson (1980) was used to isolate high-molecular-weight DNA from mature needles of *Larix* trees (DeVerno et al. 1988, 1993). The digestion of DNA samples with restriction endonucleases *Eco*RI, *Hin*dIII, and *Kpn*I was conducted according to instructions supplied by the manufacturer (BRL, Gaithersburg, Md.). Aliquots of 5 mg of each digested DNA sample were separated by electrophoresis in 0.7% horizontal agarose gels in TAE buffer (0.4 *M* TRIS-acetate, 1 m*M* EDTA, pH 8.0) at 0.5 V/cm for 16 h. DNA was transferred to Biotrans (ICN, Irvine, Calif.) nylon membranes using the LKB Vacugene Vacuum Blotting Unit Model 2016 (Pharmacia LKB Biotechnology, Uppsala, Sweden). DNA was covalently bound to the membrane by exposure to ultraviolet irradiation at an energy of 120 mJoules/cm^2 using the FB-UVXL-1000 Cross Linker (Fisher Scientific, Canada). pUC plasmid clones containing either a 2-kb *Bam*HI fragment with the wheat mitochondrial *atp*9 gene and part of the *atp*A gene (Bonhomme et al. 1989; Begu et al. 1989; Schulte et al. 1989), or a 5.6-kb *Hin*dIII fragment containing the 5′ portion of the wheat mitochondrial *nad*5 gene (Bonen et al. unpublished data, deSouza et al. 1991), encoding subunits of ATP synthase or NADH dehydrogenase, respectively, were digested with the appropriate restriction endonucleases, and inserts were separated from vector sequences by agarose gel electrophoresis. These DNA fragments were purified with Geneclean II (Bio 101 Inc., La-Jolla, Calif.) and labelled with [P^{32}]α-dCTP (Amersham, Oakville, Ont.) according to the manufacturer's instructions using the Random Primer Labelling System (BRL, Gaithersburg, Md.). Hybridizations were conducted at 60 °C with gentle shaking overnight in a solution containing 5× Denhardts (0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% BSA), 5× SSPE (0.9 *M* NaCl, 0.05 *M* sodium phosphate at pH 8.3, 0.05 *M* EDTA), and 0.2% SDS. Two hybridization washes of 20 min each were conducted in 2× SSPE with 0.5% SDS at 60 °C. Hybridized blots were exposed to Kodak XAR X-ray film in the presence of DuPont Cronex Lightning Plus intensifying screens at –70 °C for 24–72 h. The LKB UltraScan XL enhanced laser densitometer was used to analyze the intensities of the hybridizing signals on the autoradiographs.

Results

Southern hybridization experiments using wheat mitochondrial gene probes were performed on total DNA samples isolated from the needles of *Larix leptolepis, L. decidua, L. leptolepis*×*L. decidua,* and *L. decidua*×*L. leptolepis* and also on DNA isolated from somatic embryogenic cell cultures of *L. leptolepis, L. decidua, L. leptolepis*×*L. decidua,* and *L. decidua*×*L. leptolepis.* The relative intensities of the resulting hybridization signals were assessed by densitometric scanning of the autoradiographs. When the four categories of tree DNA were restricted with *Hin*dIII and probed with *atp*A/*atp*9, two major hybridizing fragments of 6.0-kb and 1.8-kb were seen (Fig. 1A, lanes 2–5), with the former being fourfold to tenfold more intense than the 1.8-kb signal (Table 1). Two hybridizing bands were consistently seen in the same relative proportions with a variety of different restriction enzymes (Fig. 1B, C, lanes 2–5); this suggests that the *atp*A/*atp*9 sequences are present in two different genomic environments, one more abundant than the other. The DNA from

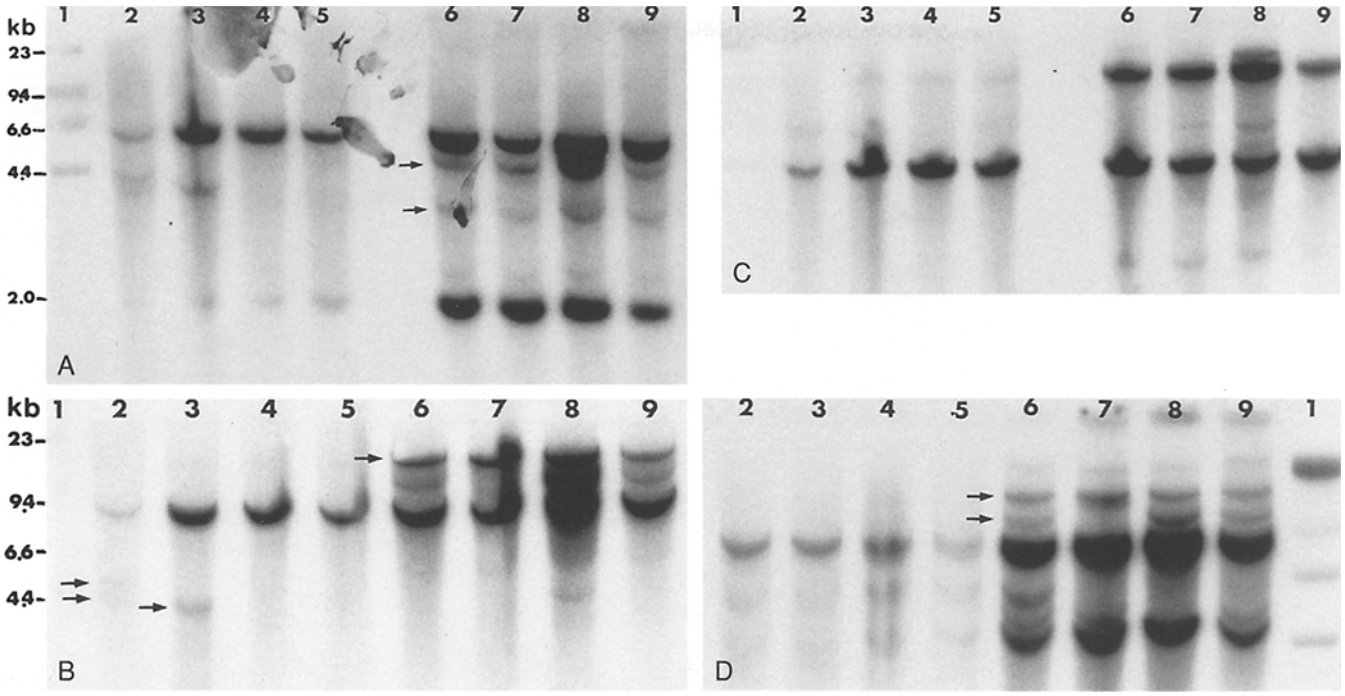

Fig. 1A–D Southern blot analysis of DNAs from tree needles and cell cultures of various *Larix* species and hybrids using wheat mitochondrial gene probes. DNAs were digested with *Hin*dIII (**A**), *Kpn*I (**B**), or *Eco*RI (**C**, **D**) and probed with *atp*A/*atp*9 (**A**, **B**, **C**) or *nad*5 (**D**). **A–D** Lane 1 Lambda *Hin*dIII digest molecular weight marker, lane 2 *L. leptolepis* DNA from tree needles. lane 3 *L. leptolepis*× *L. decidua* DNA from tree needles, lane 4 *L. decidua*×*L. leptolepis* DNA from tree needles, lane 5 *Larix decidua* DNA from tree needles, lane 6 *L. leptolepis* DNA from cell culture, lane 7 *L. leptolepis* ×*L. decidua* DNA from cell culture, lane 8 *L. decidua*×*L. leptolepis* DNA from cell culture, lane 9 *Larix decidua* DNA from cell culture

the four cell culture counterparts showed hybridization signals identical in size to those of the trees. For the *Hin*dIII digests, the ratios of the 6.0-kb:1.8-kb hybridizing fragments were only 2:1 or less, indicating an increase in the relative abundance of the genomic region containing the 1.8-kb fragment during in vitro cell culture. Similarly, when the same DNAs were digested with *Kpn*I or *Eco*RI and hybridized to the *atp*A/*atp*9 probe (Fig. 1B, C) differences were observed in the ratios of the two hybridization signals in trees versus cell culture, again consistent with amplification of one of the two mitochondrial genomic organizations containing the *atp*A/*atp*9 gene regions. Additional studies (data not shown) indicated that neither mtDNA methylation nor incomplete restriction digestion were contributing to the observed variations.

When a different mitochondrial gene probe (*nad*5) was hybridized to DNA digested with *Eco*RI (Fig. 1D) similar results were obtained in that two hybridizing fragments of 9.4-kb and 5.2-kb were observed. In the case of DNAs from *L. leptolepis* or the hybrid *L. leptolepis* × *L. decidua*, the ratio of the 9.4-kb fragment to the 5.2-kb fragment was seen to be higher in trees than in cell culture, indicating that there was an increase in the proportion of the 5.2-kb fragment during *in* vitro cell culture. However, the intensities of the hybridizing signals for the 9.4-kb and 5.2-kb fragments were approximately equal in the trees and cell cultures of *L. decidua* and the hybrid *L. decidua*×*L. leptolepis*, which would suggest that there was no change in this region of the mitochondrial genome for these two species during in vitro cell culture.

DNA samples from the hybrid tree *L. leptolepis*×*L. decidua*, somatic embryogenic cells initiated from seed of this tree, and 11 trees regenerated from these cells were digested with *Eco*RI and probed with *atp*A/*atp*9 (Fig. 2). A comparison of the ratios of intensities of the 4.1-kb and 11-kb hybridizing fragments for each DNA source showed that there was an amplification of the 11-kb fragment by 2.4-fold to 5.6-fold (Table 2) during in vitro cell culture but that the ratio of the two signals returned to one similar to that of the seed-derived tree when trees were regenerated from this culture. A two-tailed *t*-test indicated a significant difference at the 95% confidence level between the ratio of the 4.1-kb:11-kb fragments of the cell culture and regenerated trees, but no significant difference was observed between the ratio of these two fragments from the regenerated trees and the seed-derived tree.

These results demonstrate that there are quantitative changes in the relative abundance of certain mitochondrial regions during somatic embryogenic cell culture but that these changes are not always passed on to trees regenerated from these cell cultures. In addition, we observed the appearance or disappearance of certain minor hybridization signals (Fig. 1). Specifically, there appeared to be two minor hybridizing fragments in the *L. leptolepis* tree mtDNA that were not apparent in *L. leptolepis* cell culture, as well as a single fragment in *L. leptolepis*×*L. decidua* tree DNA that did not appear in *L. leptolepis*×*L. decidua*

Table 1 Ratios of relative intensities of the hybridizing signals of DNA from tree needles versus somatic embryogenic cell culture using mitochondrial gene-specific probes

	L. leptolepis		*L. leptolepis*× *L. decidua*		*L. decidua*× *L. leptolepis*		*L. decidua*	
	Tree	Cells	Tree	Cells	Tree	Cells	Tree	Cells
*(a) Eco*RI *(b) atp*A/*atp*9 *(c)* 4.1-kb:11-kb	6.1	1.3	8.8	1.1	8.8	0.9	6.6	2.0
*(a) Hin*dIII *(b) atp*A/*atp*9 *(c)* 6.0-kb:1.8-kb	5.9	2.0	6.0	0.8	10.4	2.2	3.9	1.7
*(a) Kpn*I (b) *atp*A/*atp*9 (c) 9.0-kb:15-kb	4.5	1.9	10.9	1.2	9.0	2.3	7.5	2.1
*(a) Eco*RI *(b) nad*5 *(c)* 9.4-kb:5.2-kb	4.5	1.5	3.4	2.5	1.5	1.5	1.4	1.7

Data are taken from Fig. 1 with the restriction enzymes *(a)*, probes *(b)*, and hybridizing fragments *(c)* compared,as shown in the left column.

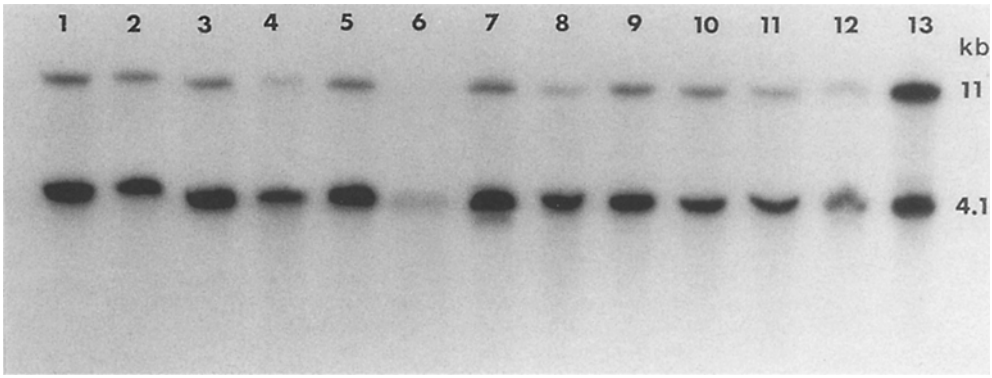

Fig. 2 Southern blot analysis of *Eco*RI-digested total DNA from trees regenerated from cell culture compared to total DNA from a tree grown from seed. Lanes 1–11: DNA from needles of 11 trees regenerated from cell culture (L287), lane 12 *L. leptolepis*×*L. decidua* DNA from mature tree needles, lane 13 *L. leptolepis*×*L. decidua* DNA from cell culture (L287)

Table 2 Ratios of relative intensities of the 4.1-kb:11-kb *atp*A/*atp*9 hybridizing signals of *Eco*RI-digested DNAs from natural trees versus somatic embryogenic cell culture and regenerated trees (RG1-11) of *L. leptolepis*×*L. decidua*. Data are taken from Fig. 2

Tree	Cells	RG1	RG2	RG3	RG4	RG5	RG6	RG7	RG8	RG9	RG10	RG11
3.2	0.97	3.0	2.4	3.7	5.6	3.3	ND	4.8	5.3	3.9	3.4	3.3

ND, not done; DNA sample degraded

somatic embryogenic cell culture (Fig. 1B, arrows lanes 2, 3). There were also minor hybridizing fragments that appeared in the cell culture that were not evident in the tree (Fig. 1A, B, D, arrows lane 6).

Discussion

Hybridization with mitochondrial gene-specific probes revealed quantitative changes in the abundance of certain forms of the mitochondrial genomic regions during somatic embryogenic cell culture of four *Larix* species. We observed that certain specific hybridizing fragments were represented at lower levels in the natural tree than in cultured cells, indicating amplification of specific molecular configurations of parts of the genome during in vitro somatic embryogenic cell culture. Similar amplifications of specific restriction fragments of the mitochondrial genome arising during in vitro cell culture have been reported for maize (McNay et al. 1984; Small et al. 1987), wheat (Hartmann *et al.* 1987), and *Chenopodium* (Dorfel et al. 1989). On the other hand, decreases in the relative proportions of restriction fragments as a result of in vitro cell culture have been reported in studies of wheat (Rode et al. 1987), maize (Gengenbach and Connelly 1981), and sugarcane (Chowdury and Vasil 1993). These minor variations may be related to shifts in the frequency of sub-stoichiometric molecules called 'sublimons' (Small et al. 1989). Changes in the mitochondrial genome during in vitro cell culture are

not always seen as demonstrated by a study of petunia (Kool et al. 1985) where no alteration of restriction fragment pattern was observed after 2 years of continuous culturing. This is similar to what we observed for the *L. decidua* × *L. leptolepis* and *L. decidua* embryogenic cultures when probed with *nad*5. However, long-term callus cultures of *Brassica campestris* were found to exhibit continuous changes in the organization of the mitochondrial genome, with various fragments increasing or decreasing in various proportions (Shirzadegan et al. 1991).

We also observed variations in some of the minor restriction fragments as demonstrated by the appearance or disappearance of specific hybridizing bands. Several novel restriction fragments appeared in the somatic embryogenic cell cultures of *Larix* while other restriction fragments present in the parent tree were not observed in the cell culture. The appearance of novel restriction fragments during cell culture has been reported for wheat (Hartmann et al. 1987) and *Brassica* (Shirzadegan et al. 1991), and a loss of restriction fragments has been reported during in vitro cell culture, for example in wheat (Rode *et al.* 1987), maize (Gengenbach and Connelly 1981), and sugarcane (Chowdury and Vasil 1993). In addition, a cell suspension culture of rice has been found to have lost one of the two copies of the apocytochrome B (*cob)* gene (Yamato et al. 1992). It is not clear whether the apparent loss and gain of restriction fragments is also due to a shift in the frequency of substoichiometric molecules, or if the fragments arise de novo.

Our analysis of mtDNA from *L. leptolepis* × *L. decidua* trees that had been regenerated from a 3-month-old somatic embryogenic cell culture indicated that the *atp*A/*atp*9 homologous region, which underwent changes during long term (3 years) in vitro cell culture, was not altered in trees regenerated from short-term in vitro culture. These regenerated trees showed a mitochondrial genome organization similar to that of the parent tree. This observation is similar to that reported for certain wheat plants regenerated from short-term (4-month-old) in vitro somatic embryogenic callus cultures in that their mitochondrial genome organization was similar to either the parental cultivar or that of the embryogenic callus (Hartmann et al. 1989). Plants regenerated from *Brassica napus* cell cultures (Kemble et al. 1988) or hybrid sugarcane embryogenic callus (Chowdhury and Vasil 1993) also showed mitochondrial genomic organizations identical to that of the parent plants. In contrast, when longer term cultures for certain wheat cultivars were used some, but not all, of the regenerated plants retained the subgenomic configuration of the in vitro culture or exhibited a novel hybridizing fragment that was not detectable in either the donor plant or cell culture (Morere-LePaven et al. 1992a, b; Hartmann et al. 1989). We did not observe any novel hybridizing fragments in the *atp*A/*atp*9 homologous region of the regenerated *L. leptolepis*×*L. decidua* tree mitochondrial genome, nor did we observe a loss of any restriction fragments. Loss of restriction fragments has been reported in maize plants regenerated from cell culture (Gengenbach and Connelly 1981) as well as in regenerated wheat plants (Rode et al. 1988). In the latter case, the loss of an 8-kb fragment of the mitochondrial genome was correlated with the loss of embryogenic potential and regenerative capacity. The similarity between the mtDNA patterns in regenerated and parental *Larix* trees in contrast to the profile of the long-term cultures *L. leptolepis*×*L. decidua* may indicate that mitochondrial genome organizational changes occurred subsequent to the regeneration of the trees used in this study. Alternatively, the in vitro somatic embryogenic culture may be composed of a mixture of cells with both normal and altered mitochondrial genomes. Therefore, the apparent reversion of the mitochondrial genome of the regenerated trees to the parental mitochondrial genome organization may be a result of the lack of regenerative capacity of those cells containing altered mitochondrial genomes, such that only "normal" mitochondrial genomes are represented in the regenerated trees.

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