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Cytogenetic analysis of reciprocal hybrids and their parents between *Larix leptolepis* and *Larix gmelinii*: implications for identifying hybrids

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Abstract We studied the parental taxa and the interspecific reciprocal hybrids between *Larix leptolepis* with *Larix gmelinii*, using classical cytogenetic methods, as well as fluorescence in situ hybridization (FISH) and genomic in situ hybridization. A high frequency (>90%) of complete bivalent formation was observed in reciprocal hybrids. Less than 10% of pollen mother cells exhibited abnormalities. The most frequent abnormalities were bridges. Multivalent chromosome associations were also observed in both reciprocal hybrids, suggesting that some chromosome interchange events did occur, and introgressions from one to the other species were possible. Intergenomic recombination indicates that genes might be readily introgressed into one species from the other in the genus *Larix*. Interspecific hybridization may be a potential method for genetic improvement in larch. FISH markers documented that the recombinant genomes of reciprocal hybrids were strictly additive and stable, indicating that FISH also might be a useful tool in *Larix* breeding.

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Introduction

Larch species ($2n=2x=24$, 1 -C =12.53 pg, http://data.kew.org/cvalues/CvalServlet?querytype=4) can easily be hybridized, and morphological and genetic studies indicate that hybridization is also widespread in natural populations (Kruklis and Milyutin 1977; Semerikov et al. 1999). Interspecific hybridization is an important process in the evolution of plant species (Rieseberg 1997) but also a source for the creation of genetic variability for breeding improvement (Desel et al. 2002). Various hybrids deriving from crosses between species of the *Larix* genus often have many attractive features such as faster juvenile growth, higher volume productivity, and greater survival than either parental species (Baltunis et al. 1998; Pâques 1992, 2002). Therefore, interspecific hybridization should provide the opportunity to access economic and other interesting traits for genetic improvement of the larch.

Larix leptolepis is an exotic reforestation species in many countries because of its fast juvenile growth and wide adaptation to severe environmental stress sites (Mikami 1988). However, this species is susceptible to various sorts of pests and diseases (Hayashi et al. 1998). *Larix gmelinii*, a main component of the Great Xingan Mountains forest in northeast of China, is also considered in larch breeding programs for its tolerance of chilling stress. Some of these

hybrid lines often combine interesting traits of both parental species, i.e., fast juvenile growth from *L. leptolepis* together with cold hardness from *L. gmelinii*. However, little is known about the genomes and meiotic chromosome behavior in interspecific *Larix* hybrids.

A better understanding of the genomes and meiotic chromosomal behavior in interspecific *Larix* hybrids will benefit larch breeding programs. The specific objective of this research was to evaluate the genome relationship between *L. leptolepis* and *L. gmelinii*, and to investigate the cytogenetics and meiotic behaviors of reciprocal interspecific hybrids between the two species.

Materials and methods

Plant materials

In 1979, reciprocal interspecific hybrids between *L. leptolepis* with *L. gmelinii* were established at Dagujia Forestry Center in Liaoning Province, China. The obtained hybrids have been grown there since. Three genotypes each species and one pair of reciprocal hybrids of each cross were selected. Seeds of their parents and young needles of hybrids were collected in 2007.

For meiotic studies, cones were collected and fixed in 3:1 absolute ethanol and glacial acetic acid at room temperature for 24 h, transferred to 70% alcohol, and stored under refrigeration until use. The anthers were dissected out and squashed on microscopic slides in the medium of 45% acetic acid for fluorescence in situ hybridization (FISH) or 1.5% carbolic acid-fuchsin solution for observations of chromosome pairing.

Chromosome preparation

Seeds were germinated in the dark on water-saturated filter paper at 25°C for 2 weeks. Root tips or young needles were submerged in 0.5% (*w/v*) aqueous colchicinefor 24 h at room temperature and fixed overnight at 4°C in 3:1 ethanol: acetic acid. Then, root tips were rinsed in 0.01 M sodium citrate/citric acid buffer and digested in 2% cellulase, 1% macerase, and 2% liquid pectinase for 1 h at 37°C. After squashing in 45% acetic acid, the coverslip was removed after freezing in liquid nitrogen, and the slides were dehydrated by consecutive immersion in 75%, 95%, and 100% ethanol, and were then air-dried and stored in a freezer at −70°C. The better preparations were identified by phase contrast Olympus BX51 microscopy.

Probe DNA labeling

Double FISH was carried out with two DNA probes. The DNA probe JDH 2-15A, containing an 8.2-kb Xho1 fragment, was kindly provided by Drs. Dai and Wu (Cornell University). The insert of 8.2 kb Xho1 fragment contains almost a full *Arabidopsis* rDNA repeat. The probe was labeled by means of high random prime with biotin-16-dUTP following the supplier's instructions (Roche, Germany), and the second probe, 5S rDNA probe, was obtained by polymerase chain reaction (PCR). The primers for 5S rDNA amplification were 5′-CGGTGCAT TAATGCTGGTAT and 5′-CCATCAGAACTCCGA-3′. The PCR program for 5S rDNA amplification consisted of 3 min at 94°C for initial denaturation followed by 30 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C. In the last cycle, the extension at 72°C was extended to 5 min. The PCR products were cloned into a plasmid vector (pGEM-1, Promega) and sequenced in both directions using an ABI 377 automatic sequencer. The 5S rDNA probe was labeled by PCR in a 50-ul reaction volume in the presence of digoxigenin-11-dUTP using the same PCR primers.

Genomic DNA was extracted from 50 to 100 mg young needles of *Larix* species using the method of Doyle and Doyle ([1990]). Genomic DNA probes were labeled with Dig High Prime (Boehringer Mannheim, Germany), according to the manufacturer's procedures. Genomic in situ hybridization (GISH) was performed on meiotic chromosomes of the F1 hybrids between *L. leptolepis* with *L. gmelinii*.

Fluorescence in situ hybridization

FISH was performed following the procedures described by Leitch et al. ([1994]) with several modifications. Slides with cell spreads were incubated in 100 ug ml^{-1} DNase-free RNase A in 2× standard saline citrate (SSC) at 37°C for 1 h, washed twice in 2×SSC for 10 min at room temperature, refixed in freshly depolymerized 4% (*w/v*) paraformaldehyde in water for 5 min at room temperature, and washed in 2×SSC for 5 min. The slides were dehydrated in a series of 70%, 95%, and 100% ethanol and then air-dried. The probe solution, consisting of labeled DNA (100 ng ml^{-1}) in a solution of unlabelled blocking DNA (5 ug ml^{-1}), 50% (*v/v*) deionized formamide, 10% (*w/v*) dextran sulphase, 0.1% (*w/v*) sodium dodecyl sulfate (SDS), and 2×SSC, was denatured at 97°C for 10 min, 10 ul; the probe mix was quenched on ice for 10 min and then loaded onto slides with cell spreads and covered with a coverslip. The slides were wrapped with preservative film and then were transferred to a humid chamber and denatured at 90°C for 10 min, then immediately put into a hybridization oven and hybridized at 37°C overnight.

Detection of hybridization

After hybridization, slides were then washed twice in 2×SSC containing 1% SDS for 5 min at 42°C, 2×SSC for

5 min at 42°C, and 2×SSC for 5 min at room temperature. The slides were washed a final time for 5 min in 2×SSC containing 0.2% Tween-20. DIG-labeled probes were detected using Rodamine diluted 1:2,000 in detection buffer. The DIG-labeled signals (5S rDNA sites) were detected using anti-DIG-FITC (Roche), and the biotin-labeled signals (45S rDNA sites) were detected using strepetavidin-Cy3 (Sigma). The antibody binding reaction was carried out at 37°C in 5% bovine serum albumin (BSA) in 2×SSC containing 0.2% Tween-20 for about 1 h. The slides were washed three times in 5% BSA in 2×SSC containing 0.2% Tween-20 for 5 min each at 37°C. Finally, chromosomal DNA was counterstained for 3–5 min in 0.5 ug/ml of 4′,6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline (PBS; pH 7.4), rinsed in 1×PBS, and mounted with anti-fade solution (Vector Laboratories Inc., Burlingame, CA, USA).

All images were captured with an Olympus BX51 phase/fluorescence microscope equipped with a cooled CCD camera and processed by Adobe Photoshop 7.0 software.

Results

Chromosomal distribution of rDNA loci

Both the interspecific hybrid and its reciprocal contained the expected chromosome number ($2n=2x=24$; Fig. 1a, b). FISH localization of the 45S rDNA genes differentiated the two parental species by the number of rDNA sites. *L. leptolepis* had two pairs of 45S rDNA signals (Fig. 1a), whereas *L. gmelinii* had three pairs of 45S rDNA signals (Fig. 1b). When the 45S rDNA probe was hybridized to mitotic cells of the two hybrids, five fluorescence signals were observed, as expected, in all accession cells of these two hybrids. Thus, we concluded that the FISH signals of 45S rDNA repetitive sequences in both parents were different, and in the hybrids, the signals were exactly additive (red signals in Fig. 1c, d).

Only one 5S rDNA locus was detected within each genome of the two parental and hybrid species and was co-localized with a 45S rDNA, giving a strong double-hybridization signal that allowed this chromosome to be readily identified (green signals in Fig. 1). The distribution patterns of the two types of rDNA genes appeared similar in the three genomes, indicating conservation of the chromosomal position of the rDNA within both parental genomes.

Meiotic behavior

Cytological analysis of meiosis in the parental species revealed over 97% of the meiocytes showed regular bivalent chromosome pairing; however, several meiotic irregularities, such as ascending, anaphase bridges, and multivalent pairing, were occasionally observed, whose type and frequency are presented in Table 1.

The general features of the meiotic behavior of the reciprocal hybrids are also summarized in Table 1. Irrespective of the maternal parents, the patterns of meiotic configurations of reciprocal hybrids were fairly similar (Table 1). A high frequency (>90%) of complete bivalent formation was observed in reciprocal crosses (Fig. 2a, b), indicating that the genomes of *L. leptolepis* and *L. gmelinii* were highly homologous. Our results, moreover, showed that less than 10% of pollen mother cell exhibited abnormalities, such as multivalents (Fig. 2c, d), univalents (Fig. 2e, f), and irregular chromosome segregation (ascending, laggard, and bridge; Fig. 2g–i). Multivalents were relatively rare (Fig. 2c), and some trivalents have a "fryingpan" (a ring bivalent connected to a rod univalent) appearance (Fig. 2d), indicating that some chromosome interchange events did occur. A few unpaired univalents were observed (Fig. 2e, f) owing to the lack of chromosome pairing. In anaphase-I, they moved randomly to opposite poles (Fig. 2g–i). Irregular chromosome segregation was the main problem found in meiosis I. The most frequent configuration was chromatin bridges in anaphase-I (Fig. 2g), which accounted for 14% and 16% of the anaphase-I cells of *L. leptolepis*×*L. gmelinii* and *L. gmelinii*×*L. leptolepis*, respectively (Table 1). Due to the high frequency of bridges, segregation was irregular at anaphase-I (Fig. 2h, i). These irregular misdivisions may lead to telocentrics or isochromosomes. Ascending and laggard chromosomes were also observed at telophase-I in some cells (Fig. 2g–i), which could result in gametes with various chromosome numbers because of unequal chromosome migration, segregation, and allocation in daughter cells. However, the imbalanced distribution of chromosomes in anaphase-I did not persist up to the second division, in which chromatids were split normally into daughter nuclei (data not shown), suggesting a strong selection against abnormal meiotic products in early stages.

To better characterize the genomic changes in hybrids, we traced the behaviors of the rDNA containing chromosomes as a cytogenetic markers for potential genomic processes of these reciprocal hybrids by FISH. In the present work, we found homoeologous chromosomes exclusively forming bivalents in most meiotic cells of these reciprocal hybrids based on the distribution of 45S and 5S rDNA at diakinesis/metaphase-I (Fig. 3a, b), suggesting that homoeologous bivalents were formed at a relatively high rate. Regular homoeologous pairing in the hybrids insured proper segregation of chromosomes. Figure 3, c and d, show that two pairs of chromosomes possessing the

Fig. 1 Physical mapping of
45S rDNA (*red*) and 5S rDNA
(*green*) to somatic metaphase
chromosomes, and chormo-
somes were counterstained
with DAPI (*blue*). **a** *Larix
leptolepis*. **b** *Larix gmelinii*.
c *L. leptolepis×L. gmelinii*.
d *L. gmelinii×L. leptolepis*.
Scale bar=15 um
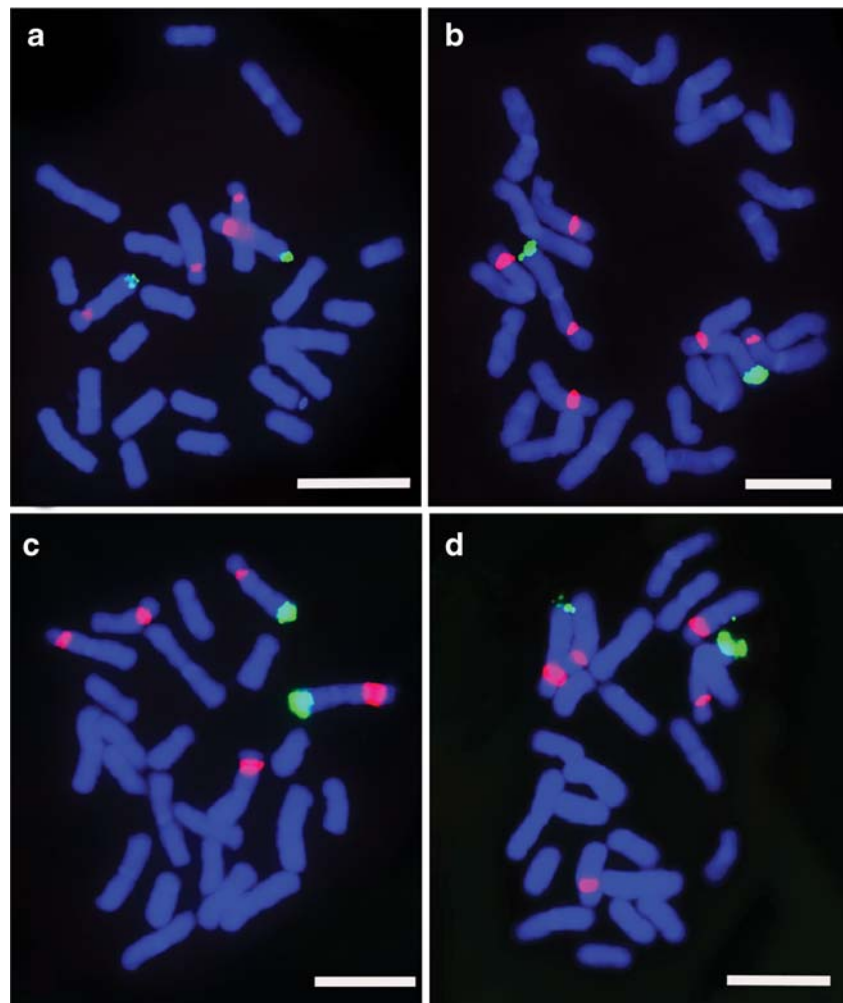
rDNA signals divided regularly into the two daughter cells at anaphase-I. At anaphase-II, the sister chromatids separated and moved towards opposite cell poles (Fig. 3e). Based on the rDNA signals, irregular segregations were also verified. Figure 3, f and g, show one pair of rDNA bearing chromosome forming a bridge at anaphase-I in *L. leptolepis×L. gmelinii* and *L. gmelinii×L. leptolepis*, respectively. Furthermore, the imbalanced distribution of

Table 1 Numbers of pollen mother cells analyzed and the percentage of cells with specific meiotic abnormalities

Meiotic types	*L. leptolepis*		*L. gmelinii*		*L. leptolepis×L. gmelinii*		*L. gmelinii×L. leptolepis*	
	D/M	A-T	D/M	A-T	D/M	A-T	D/M	A-T
AC (%)	0.8	–	o.6	0	1.2	1.8	2.2.	1
LAG (%)	0	–	0	0	–	2.1	0.6	2
BRI (%)	–	4	–	0	–	14.5	–	16
BIV (%)	97.3	–	97.5		94.6	–	93.2	
UIN (%)	0	–	0	0	2.0	–	2.0	–
MUL (%)	2.7	–	2.5	0	3.4	–	4.8	–
Total cells analyzed	244	40	261	50	248	138	147	50

D diakinesis, *M* metaphase, *A* anaphase, *T* telophase, *AC* ascending chromosomes, *LAG* laggard chromosomes, *BRI* bridge, *UIN* univalent, *MUL* multivalent *BIV* bivalent

Fig. 2 Meiotic cells in pollen mother cells (PMCs) of reciprocal interspecific hybrids between *Larix leptolepis* with *Larix gmelinii* (stained with carbolic acid-fuchsin). **a** Diakinesis of a PMC from *L. leptolepis*×*L. gmelinii* showing 12 bivalent configurations. **b** Metaphase-I of a PMC from *L. gmelinii*×*L. leptolepis* showing 12 bivalent configurations, which are arranged normally at the metaphase plate. **c** Diakinesis of a PMC from *L. leptolepis*×*L. gmelinii* showing quadrivalent (*arrow*). **d** Diakinesis of a PMC from *L. gmelinii*×*L. leptolepis* showing a frying pan-shaped trivalent (*arrow*). **e, f** Metaphase-I of a PMC from *L. leptolepis*×*L. gmelinii* and *L. gmelinii*×*L. leptolepis*, respectively, showing two univalent, which are scattered outside the plate. **g** Anaphase-I of a PMC from *L. leptolepis*×*L. gmelinii* showing bridge and fragment (*arrows*). **h** Anaphase-I of a PMC from *L. gmelinii*×*L. leptolepis* showing laggard chromosome (*arrow*). **i** Telophase-I of a PMC from *L. leptolepis*×*L. gmelinii* showing laggard chromosome (*arrow*). Scale bar=15 um
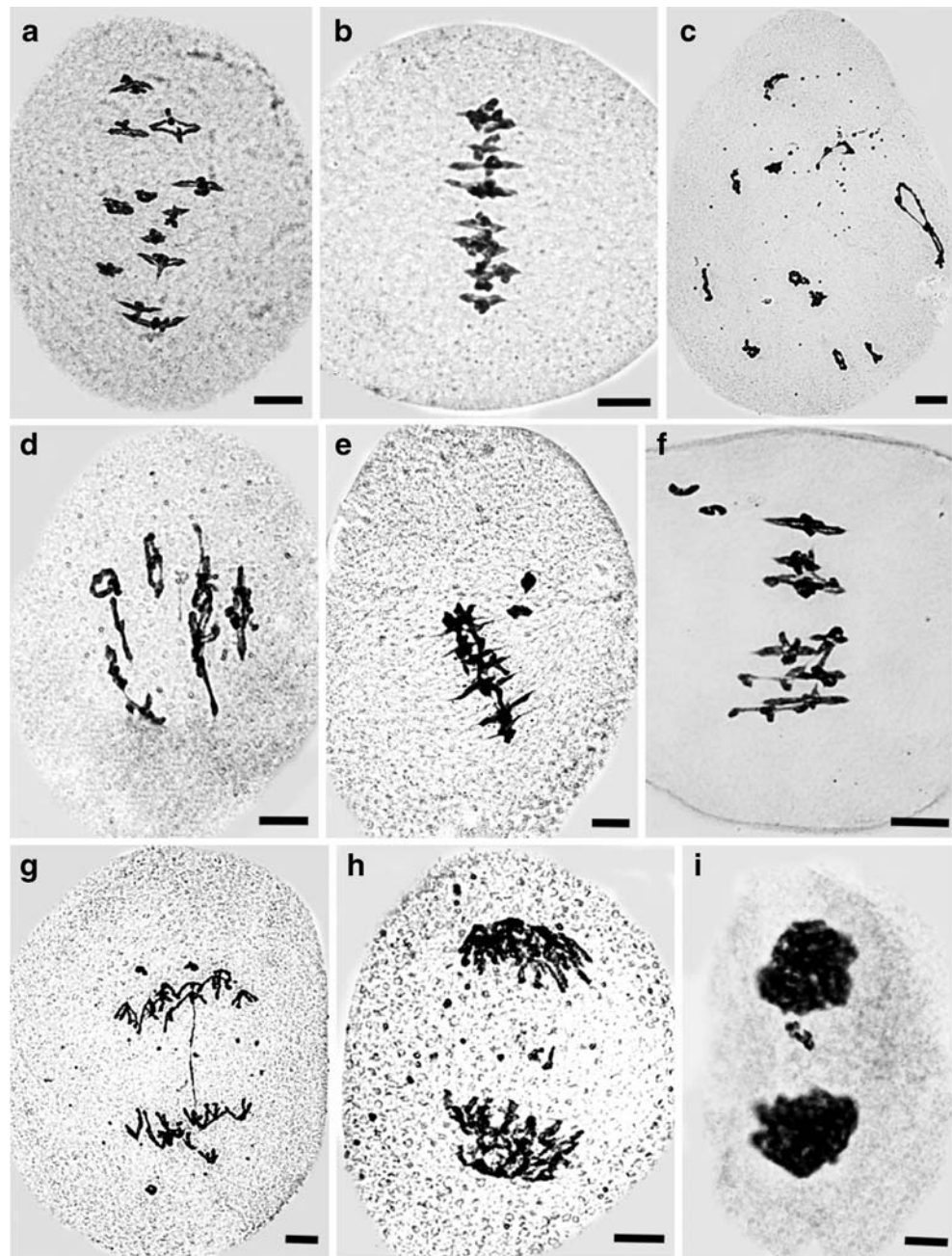
chromosomes carrying 45S rDNA were observed in early telophase-I in some cells (Fig. 3h).

Although the rDNA loci as physical markers provided convincing evidence for matching chromosome homologues between these species, the same GISH conditions failed to discriminate the chromosomes of *L. leptolepis* and *L. gmelinii* from each other in meiotic chromosome preparations of these reciprocal hybrids (Fig. 3i). Thus, we were not able to assess the extent of homologous and homoeologous pairing.

Discussion

Genome relationships between *L. leptolepis* and *L. gmelinii*

Evidence from classical and molecular systematics, ecology, and crossing relationships all indicate *L. leptolepis* and *L. gmelinii* are closely related sister species (Semerikov and Lascoux 1999; Khatab et al. 2008). This was also confirmed in the present study, where the high frequency of homoeologous chromosome pairing in reciprocal hybrids

Fig. 3 Fluorescence in situ hybridization to chromosomes of meiotic nuclei of reciprocal interspecific hybrids; 45S rDNA loci and 5S rDNA loci are identified by *red* and *green* signals, respectively. **a**, **b** Meiotic metaphase-I of a pollen mother cell (PMC) from *Larix leptolepis×Larix gmelinii* and *L. gmelinii×L. leptolepis*, respectively, showing rDNA bearing-chromosomes exclusively formed bivalents. **c**, **d** Two pairs of rDNA bearing-chromosomes regularly separated into opposite sides of the cell in *L. leptolepis×L. gmelinii* and *L. gmelinii×L. leptolepis*, respectively. **e** Meiotic telophase-II of a PMC from *L. gmelinii×L. leptolepis* showing sister chromatids regularly divided. **f** Meiotic anaphase-I of a PMC from *L. leptolepis×L. gmelinii* showing 45S-5S rDNA bearing-chromosomes formed bridge. **g** Meiotic anaphase-I of a PMC from *L. gmelinii×L. leptolepis* showing 45S rDNA bearing-chromosomes formed bridge. **h** Meiotic anaphase-I of a PMC from *L. leptolepis×L. gmelinii* showing unequal separation of 45S rDNA bearing-chromosomes. **i** Genomic in situ hybridization on meiotic metaphase chromosomes of the interspecific species *L. leptolepis×L. gmelinii*, using labeled genomic DNA from *L. leptolepis* as probe (detected with strepetavidin-Cy3, in *red*). Chromosomes were counterstained with DAPI, in *blue*. Scale bar=15 um
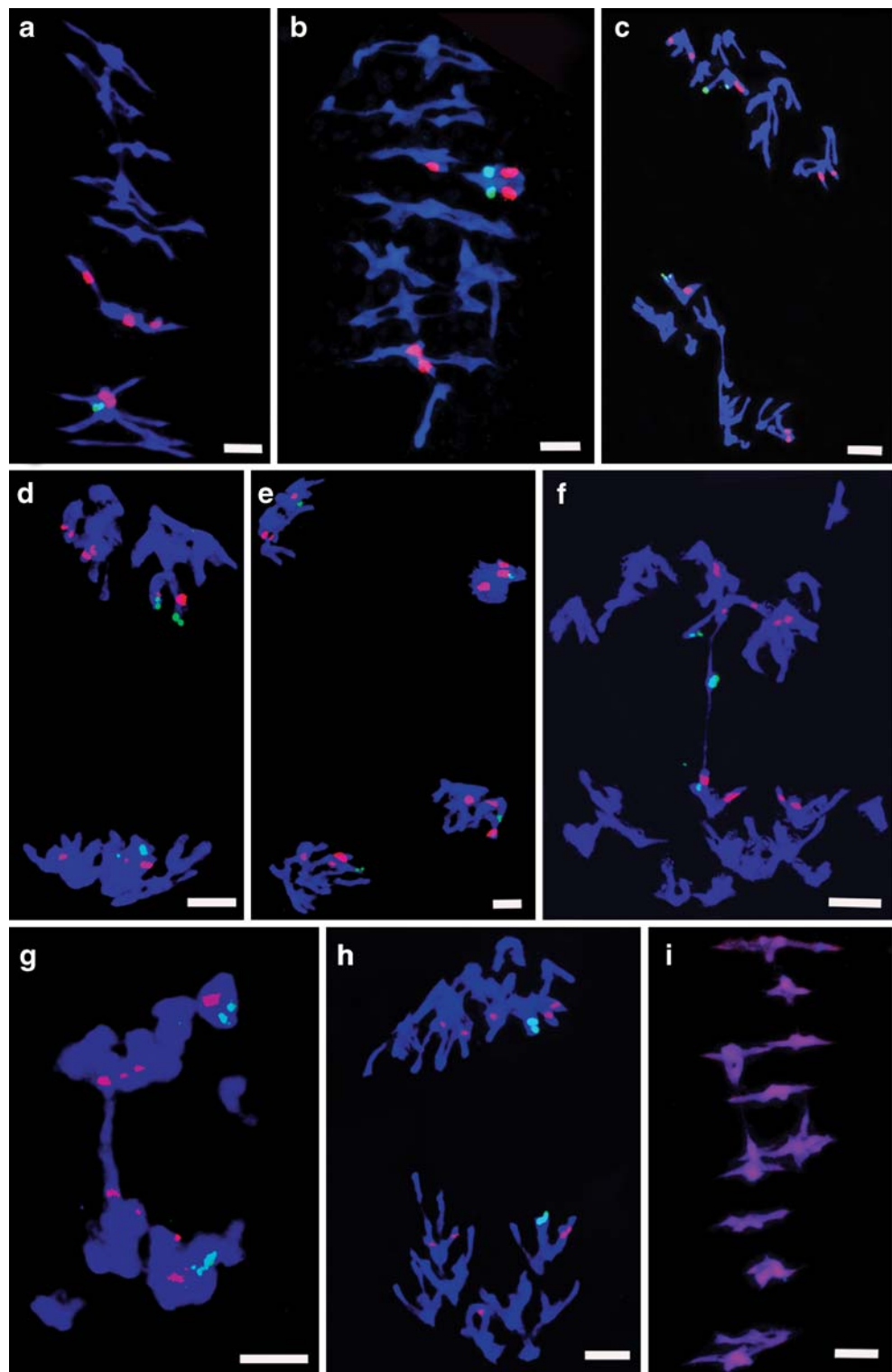
indicated that they shared a high degree of homology. This conclusion was consistent with GISH analysis, in which standard GISH conditions were insufficient to distinguish the recombinant genomes of the hybrids from each other. It was suggested that homology between *L. leptolepis* and *L. gmelinii* was relatively high at the sequence level, at least in terms of dispersed repetitive sequences. In addition, we observed the same distribution patterns and the numbers of 5S rDNA loci in the two parental species, as well as the similar chromosomal location of 45S rDNA loci. Giving

that these species also have a number of cytological features in common (Liu et al. 2006), we therefore assumed that the chromosomes of these two parental species within *Larix* were essentially collinear and homologous. This results presented here also suggest that the overall structures of the genomes of these species have not changed excessively during the course of evolution.

In contrast, the numbers of 45S rDNA loci were evidently different between the two parental species, resulting in physically distinct chromosome sets.

Relevance of interspecific hybrids to breeding

Interspecific hybridization plays an important role in the creation of genetic variability for breeding improvement (Desel et al. 2002). The efficiency of interspecific crosses depends on the level of genetic and structural relatedness between the implicated genomes as high levels of genome similarity are required to ensure appropriate chromosome pairing and genetic recombination (Leflon et al. 2006). Meiotic recombination is essential for information from the parental chromosomes to be combined into new genetic entities that can be passed to the next generation (Puchta and Hohn 1996). In principle, more frequent introgressions are expected when the recombinant genomes share higher levels of similarity (Panestos and Baker 1967; Boudry et al. 1993). Our results showed that the genomes of *L. leptolepis* and *L. gmelinii* exhibit high homology at the DNA sequences, which allowed regular pairing and recombination between homoeologous chromosomes at relatively high rate in their hybrids. Cytological observations clearly demonstrated that the two hybrids generally showed regular bivalent formations, indicating that meiotic recombination did occur between the two parental sets of homoeologous chromosomes. In addition, quadrivalents and trivalents occasionally formed in these two reciprocal hybrids suggested that some chromosome interchange events did occur. We also observed several meiotic abnormalities in these hybrids; however, these would be unlikely to not greatly influence breeding or introgression due to their low frequency and a strong selection against abnormal meiotic products in early stages of development.

In conclusion, *Larix* hybrids may have frequent inter-genomic recombination events, and that genes may be readily introgressed into one species from the other in the genus *Larix*. Interspecific hybridization may be a potential method for genetic improvement in larch.

Implications for identifying hybrids

Breeding programs involving interspecific hybrids should rely on an accurate system of identification of parental species and certification of their putative hybrids to ensure the delivery of the desired materials and to obtain the predicted genetic gains (Gros-Louis et al. 2005). Although some morphological traits allow identification of *Larix* species at the mature stage, differentiation is not always possible to distinguish either hybrid seeds or juvenile plants. Therefore, there is a need for the development of reliable markers to distinguish parents and their hybrids. Certain molecular markers have already been developed for some larch species (Bergmann and Ruetz 1987; Scheepers et al. 2000; Acheré et al. 2004; Gros-Louis et al. 2005; Moriguchi et al. 2008), for which hybrids are difficult to detect on the basis of morphology at early stage. However, such studies depend on wide screening for fixed molecular markers or sequences from which the species in question can be diagnosed. It is often time-consuming to find reproducible species-diagnostic molecular markers among larch species as they share a high degree of sequence homology (Nkongolo and Klimaszewska 1995). Another factor suggested to be responsible for this lack of efficiency is genomic reshuffling due to hybridization, which would obscure true interpretation of molecular markers in hybrids.

In contrast, FISH of metaphase chromosomes can enable the unequivocal determination the genomic constitution of hybrid plants (Desel et al. 2002; Markova et al. 2006). Physical mapping by FISH of the tandemly repeated genes encoding the 18S-5.8S-25S ribosomal RNA and 5S ribosomal RNA genes has provided a number of useful chromosome landmarks in larch trees (Lubaretz et al. 1996; Liu et al. 2006). These studies showed that the rDNA organization in *Larix* varies among species. Thus, if the hybrid has an additive character, molecular cytogenetics of the rDNA genes would offer a relatively efficient way to differentiate varieties in seed orchards or a special breeding program at the level of chromosomes.

The present study reported experiments to assess whether FISH could discriminate hybrids between *L. leptolepis* and *L. gmelinii*, which appeared to be especially useful for two reasons. First, no prior genetic information on the species analyzed is required. Second, chromosomal in situ DNA hybridization (ISH) allows the physical sites of DNA sequences to be visualized directly, without regard to polymorphism or coding ability (Crane et al. 1993). Therefore, FISH can serve as a useful tool for identifying hybrids in future breeding programs, as well as a means to analyze the genomes of the genus *Larix*.

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