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Multicolor fluorescence *in situ* hybridization with combinatorial labeling probes enables a detailed karyotype analysis of *Larix principis-rupprechtii*

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ABSTRACT

The chromosomes (2n = 2x = 24) of *Larix principis-rupprechtii* are composed of six pairs of large metacentrics and six pairs of medium-sized submetacentrics. The identification of homologous pairs is hampered by their high degree of similarity at the morphological level in each group. As one of the most extensively used methods in molecular cytogenetics producing chromosome landmarks, fluorescence *in situ* hybridization (FISH) has significantly facilitated karyotype construction, especially in species with morphologically similar chromosomes. This study developed a simple but effective use of combinatorial labeling probes to distinguish chromosomes of *Larix principis-rupprechtii* by multicolor FISH. Three highly repetitive sequences in *Larix* were selected: 25S rDNA hybridized at all of the secondary constrictions of two pairs of metacentrics and the largest pair of submetacentrics; 5S rDNA hybridized at subtelomeric sites of one pair of metacentrics that also harboured 25S rDNA on different arms; LPD family sequences are tandem repeats hybridized at proximal regions of 22 chromosomes. The three different probes were labeled with only two different labels, hybridized to metaphase chromosomes of *Larix principis-rupprechtii,* simultaneously visualized, and unequivocally distinguished in a single FISH experiment. These multicolor FISH marks largely improved the karyotype analysis of *Larix principis-rupprechtii*.

Key terms: combinatorial labeling probes, fluorescence *in situ* hybridization, karyotype, *Larix principis-rupprechtii*, LPD repeats, 25S and 5S rDNA

Larix principis-rupprechtii is a perennial deciduous arbor mainly planted in north of China. The diploid chromosome number (2n = 2x = 24) is very conserved in the family of Pinaceae, with little karyotype differentiation between species (Friesen et al., 2001). The chromosomes of *Larix principis-rupprechtii* constitute a bimodal karyotype in which they are divided into two groups, a group of twelve large metacentrics and the other group of twelve medium-sized submetacentrics (Zhang et al., 1985). However, in each group the identification of homologous pairs is hampered by similar length and slight differences at the morphological level. On the other hand, chromosome landmarks produced by fluorescence *in situ* hybridization (FISH) with various repetitive sequences as probes make the identification of chromosomes easier. This strategy has been applied successfully in several species of other genera in Pinaceae, e.g. *Pinus* (Hizume et al., 2002b; Liu et al., 2003) and *Picea* (Brown et al., 1998; Siljak-Yakovlev et al., 2002; Vischi et al., 2003). In *Larix*, an improvement has been proposed by Lubaretz et al. (1996) with rDNA and telomeric repeats probes, but these probes are inadequate for distinguishing every chromosome pair. Hizume et al. (2002a) identified and characterized the LPD family as tandemly repetitive DNA sequences in *Larix leptolepis*, and they concentrate in

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proximal regions of most chromosomes of *Larix* so that the LPD repeats could be a novel probe in chromosome identification of *Larix*.

The commercial availability of a range of different fluorophores has enabled the simultaneous imaging of several target sequences in one experiment. In plants, however, traditionally a maximum of three fluorophores are used simultaneously, which emit either blue (amino-methylcoumarin; AMCA), green (fluorescein and derivatives, e.g. FITC) or red light (Texas red, rhodamine, cyanine3; Cy3). Routine counterstaining of chromosomes with for example DAPI, YOYO or propidium iodide, effectively reduces this number since emission wavelengths of these counterstains interfere with emission wavelengths of the commonly used fluorophores. Combinatorial labeling, which incorporates more than one fluorophore into a probe, is a means by which a greater number of chromosome targets may be discriminated. It was developed for human cytogenetics and is considered as a breakthrough in clinical diagnostics (Ried et al., 1992). In plants, Jiang et al. (1995) successfully mapped three different bacterial artificial chromosomes in the rice genome with this approach and Hasterok et al. (2002) improved this method in *Secale cereale* and *Triticum aestivum*. To date, combinatorial labeling of probes has scarcely been used for molecular cytogenetics of gymnosperms.

In this study, we developed a simple and efficient technique of multicolor fluorescence *in situ* hybridization in *Larix principis-rupprechtii*, which allows the use of probes labeled with two standard labels to map simultaneously three different DNA sequences along somatic chromosomes. Furthermore, a more detailed karyotype of *Larix principis-rupprechtii* could be constructed with these landmarks produced by the multicolor FISH.

Seeds of *L. principis-rupprechtii* were collected in a planted larch forest at Dagujia Forestry Centre in Liaoning Province, China. The seeds were sown in sterilized sand in a pot, and 10-14 days later the primary root tips were collected for chromosomal analysis. The root tips were treated with 0.05% colchicine at 20ºC for 14 h, fixed in chilled Carnoy's fixative (ethanol: acetic acid = 3: 1, v/v) overnight. Fixed root tips were macerated in an enzyme mixture containing 2% cellulase ("Onozuka" R-10, Kinki Yakult) and 1% pectolyase (*Aspergillus niger,* Serva) in citrate/citric acid buffer (pH 4.5). The meristematic cells were squashed under a coverslip on a glass slide and air dried after the coverslip was removed with liquid nitrogen.

Total genomic DNA was extracted from young leaves of *L. principis-rupprechtii* by the CTAB method (Murray and Thompson, 1980). A 2.3 kb subclone of the 25S rDNA coding region of *Arabidopsis thaliana* (Unfried and Gruendler, 1990) was labeled with Biotin-16-dUTP (Roche) by randomly-primed DNA synthesis according to manufacturer's instructions. The 5S rDNA clone pTa794 (Gerlach and Dyer, 1980) was amplified and labeled with digoxigenin (DIG)-11-dUTP (Roche) using PCR with universal M13 primers. The LPD family sequence was amplified from total genomic DNA of *L. principis-rupprechtii* by PCR using the primers LPD-f: 5'-CATGCATCCGGAAATAAGAA-3' and LPD-r: 5'-CGCAAACTATAGAAAGTCGT-3' (Hizume et al., 2002a), and the product was purified and labeled the same way as 25S rDNA but simultaneously and in equal proportions with digoxigenin-11-dUTP and biotin-16-dUTP.

FISH procedure was carried out as described previously (Liu et al., 2005). Briefly, chromosomal DNA was denatured at 70ºC for 2 min in 70% formamide, 2×SSC. Probes dissolved in 2×SSC, 5% dextran sulfate, and 50% formamide were denatured at 94ºC for 10 min. The hybridized probes were detected with anti-DIG-fluorescent-conjugate (FITC, Roche) and Strepetavidin-Cy3 (Sigma). The slides were counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma) and mounted in Vectashield mounting medium (Vector Laboratory). The hybridization signals were visualized and recorded using Nikon 80i fluorescence microscope and a cooled CCD camera (SPOT, Diagnostic Instruments) equipped with the version

4.0.8 of the Spot software (Diagnostic Instruments). Ten metaphases were scored. Images were processed using Adobe Photoshop 7.0.

The length of short and long arms was measured, and arm ratio (length of long arm/length of short arm) and relative length were estimated for each chromosome. The nomenclature system for the position of the centromere followed that of Levan et al. (1964). Complementary chromosomes were arranged in order of decreasing length.

Figure 1 shows the results of simultaneous triple FISH to the somatic metaphase chromosomes of *L. principis-rupprechtii*. Figure 1A-C show results from single band pass filters in blue, red and green channels, respectively, whilst Figure 1D present electronically superimposed images combining the three individual channels. In Figure 1B, 28 distinct red signals with different size and intensity indicated the hybridized loci of biotin-labeled probes, six of which marked the

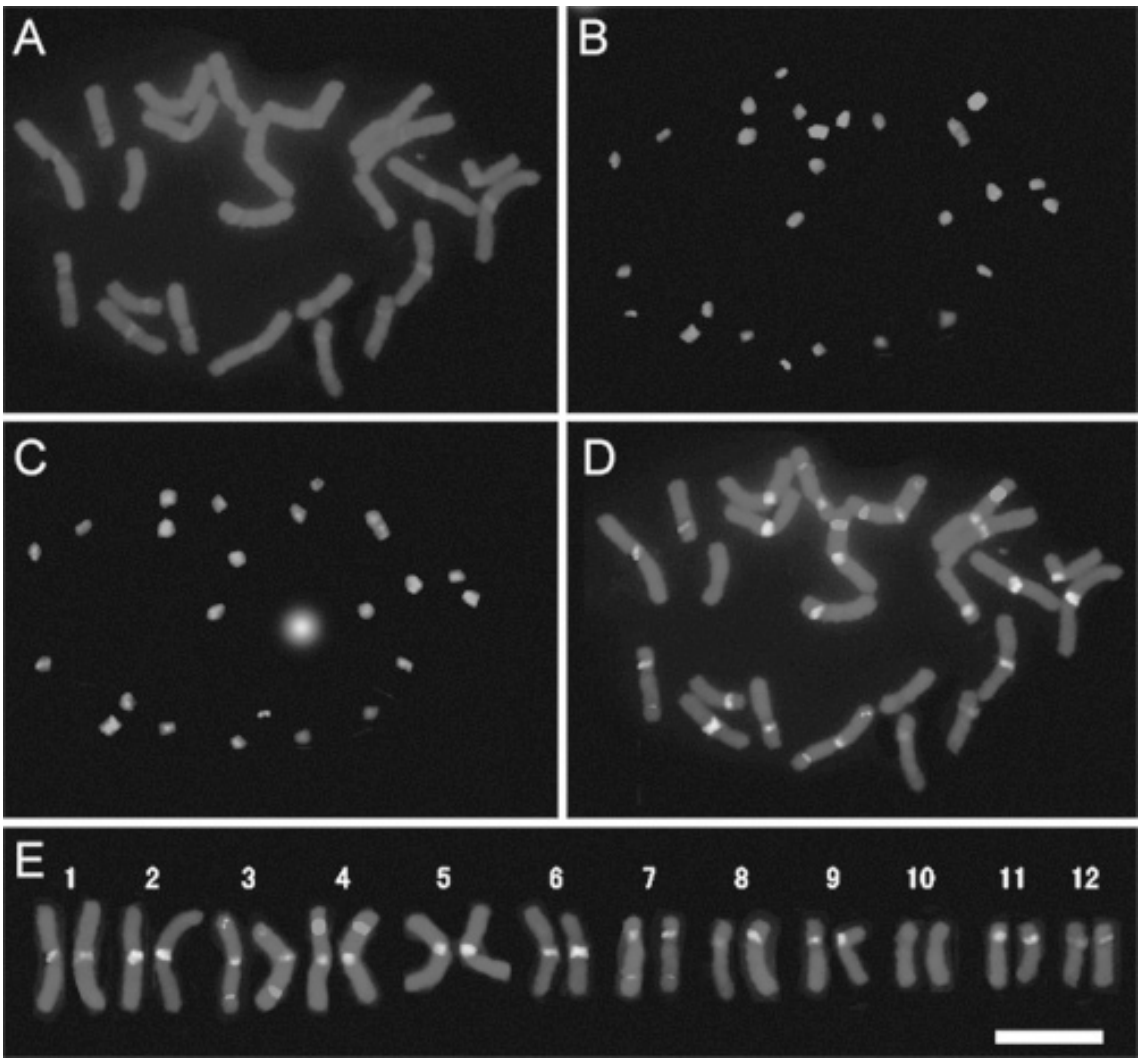

Figure 1: Simultaneous triple FISH to somatic metaphase chromosomes of *L. principis-rupprechtii*. (A) Chromosome spread counterstaining with DAPI (blue). (B) Red hybridized signals from 25S rDNA and LPD repeats. (C) Green hybridized signals from 5S rDNA and LPD repeats. (D) Chromosome spread simultaneously with 25S rDNA, 5S rDNA, and LPD repeats loci in pink, green, and white color, respectively. (E) Homologous chromosome pairing and ordering. Bar. 5 μm.

25S rDNA loci and the other 22 were produced by LPD repeats probe. In Figure 1C, 24 distinct green signals indicated the hybridized loci of DIG-labeled probes, two tiny ones represented the 5S rDNA loci and the other 22 produced by LPD repeats probe were the same in size and intensity with the red signals of the same location in Figure 1B. After composed Figure 1A-C, signals with three different color were shown at chromosome spread (Fig 1D). The six pink signals were the 25S rDNA red signals against the blue DAPI counterstaining and located at the secondary constrictions of two pairs of metacentrics and the largest pair of submetacentrics. The two tiny green 5S rDNA signals located at the subterminal regions of one pair of metacentrics that also harbored a 25S rDNA locus on different arms. Since the combinatorial labeling LPD repeats fluoresced red and green simultaneously (Fig 1B and C), they showed white signals in proximal regions of 22 chromosomes in the composite image against the blue DAPI counterstaining of chromosomes. Thus, the multicolor discrimination of three different DNA sequences was accomplished within a single FISH experiment with only two labels.

The chromosomes of *L. principis-rupprechtii* in a given cell can be easily divided into two groups according to their length and centromere positions. Furthermore, with the help of these FISH signals above, we could complete the identification of homologous chromosome pairs within each group easily. First, by the 25S and 5S rDNA signals, three pairs of chromosomes could be identified unambiguously, as a double marked metacentric pair with both 25S and 5S rDNA loci on its different arms, a metacentric pair with only 25S rDNA loci, and a submetacentric pair with 25S rDNA loci on its long arms. Although DAPI bands concentrated in proximal regions of most chromosomes are displayed after DAPI counterstaining (Fig 1A), the white LPD repeats signals representing the same region exclusively (Hizume et al., 2002a) show more contrast to the blue background of chromosomes and make the paracentromeric regions of all chromosomes but one submetacentic pair clear. Hizume et al. (1994) observed only 20 DAPI bands in *L. principis-rupprechtii* with two fewer at submetacentric pairs than those in our study, maybe because some DAPI bands are too faint to be identified while LPD repeats FISH signals are more obvious (Fig 1A and D). The chromosomes with similar hybridized signal size and intensity of LPD repeats could be identified as homologous pairs. Thus, in addition to the three pairs of chromosomes that have been identified by rDNA probes, other eight chromosomes pairs bearing distinct LPD repeat signals and one pair without FISH signal were found. Then the statistical analysis of microscopic measurements of

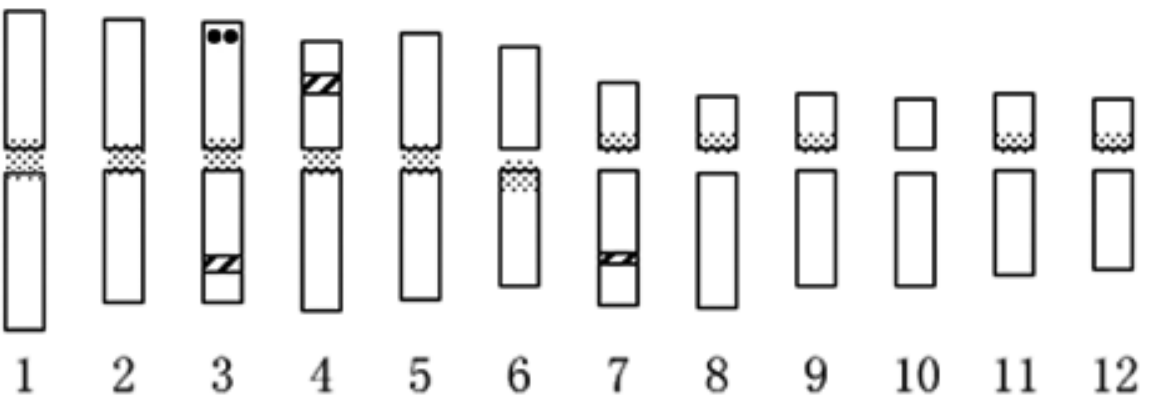

Figure 2: Idiogram of *L. principis-rupprechtii* with 25S rDNA (slash area), 5S rDNA (filled circle), and LPD repeat (dot) loci.

chromosomes was carried out. Complementary chromosomes were arranged in order of decreasing length as shown in Figure 1E and their idiogram in Figure 2. According to the ordering, the chromosome pair bearing both 25S and 5S rDNA loci is No. 3. Chromosome 4 and 7 bear 25S rDNA loci at their short arm and long arm, respectively. The only pair without any FISH signal is chromosome 10. The karyotype data including the length of short and long arms, arm ratio (length of long arm/length of short arm), and relative length estimated for each chromosome were listed in Table I. Since the homologous chromosome pairing is more credible with the assistance of multicolor FISH signals, the karyotype data could be more accurate than those obtained from conventional karyotype. However, the length of the chromosomes within the large group and the medium-sized group each are so close that some ordering could be mistaken. This could even happen for the identification of long/short arm of the metacentrics, especially for chromosome 2 and 3. To solve this problem, more chromosome landmarks are needed, and then, the detailed karyotype could be constructed not only according to the length of chromosomes.

Simultaneous *in situ* hybridization of more than two different DNA probes to the same chromosome preparation is informative and could help us to complete the construction of detailed karyotypes of *Larix,* but there are some noticeable technical problems. First, it is important to ensure that the emission spectra of the probes are sufficiently well separated from each other, and from the counterstaining of the chromosomes. We selected Cy3 and FITC as reporters, which fluoresce in the red channel and green channel, respectively. They are distinct from each other and also from the blue background of chromosomes produced by DAPI. Secondly, we should be cautious with probes whose loci are totally or in part overlapped. If two probes are supposed to be detected by a single color but they co-localize at the same loci of metaphase chromosome, they will display composite color and that will foster confusion with the third probe of combinatorial labeling. Although it is possible for more than three different probes to be detected simultaneously through combinatorial labeling with more labels, or even by varying ratios of different labels for each probe, the non-standard emission filters and higher sensitivity CCD camera are needed.

TABLE I

Measurements of somatic metaphase chromosomes of *L. principis-rupprechtii* (2n = 24). *m* metacentric, *sm* submetacentric

Chromosome number	Relative length of short arm	Relative length of long arm	Relative length	Arm Ratio	Chromosome type
1	5.348	6.113	11.551	1.124	m
2	5.017	5.101	10.118	1.017	m
3	4.975	5.101	10.076	1.025	m
4	4.216	5.438	9.654	1.290	m
5	4.553	5.017	9.570	1.102	m
6	4.089	4.469	8.558	1.093	m
7	2.614	5.270	7.884	2.016	sm
8	2.150	5.312	7.462	2.471	sm
9	2.234	4.511	6.745	2.019	sm
10	1.981	4.469	6.450	2.256	sm
11	2.204	4.089	6.113	2.020	sm
12	1.981	3.836	5.817	1.936	sm

In this study, we demonstrated above that in gymnosperms with large chromosomes like *Larix* species, combinatorial labeling of probes offers a simple, inexpensive and efficient way of discerning more chromosome targets with the same number of fluorophores in one FISH experiment. By this technique and based on several appropriate probes, we may construct FISH karyotypes of *L. principis-rupprechtii* and other *Larix* species, and that would provide us more detailed information for karyotype analysis and even for comparison among species.

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