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Karyotyping of three Pinaceae species via fluorescent *in situ* hybridization and computer-aided chromosome analysis

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Abstract The positions of 18/25S rRNA genes, 5S RNA genes and of *Arabidopsis*-type telomeric repeats were localized by fluorescent *in situ* hybridization (FISH) on the chromosomes of three coniferous species; *Picea abies*, *Larix decidua* and *Pinus sylvestris*, each with 2n=24 chromosomes. Computer-aided chromosome analysis was performed on the basis of the chromosome length, the arm length ratio and the position of the hybridization signals. This enabled the chromosomes of the Norway spruce, 4 chromosomes of the European larch and 3 of the karyotype of the Scots pine to be individually distinguished. With respect to the chromosomal positions of rDNA and 5S rDNA loci, chromosome pair I of *P. sylvestris* is suggested to be homoeologous to pair II of *P. abies*, while another chromosome pair of *P. sylvestris* might be homoeologous to chromosome pair III of *L. decidua*.

Key words Pinaceae · rDNA · 5S DNA · FISH · Karyotyping

Introduction

Picea abies, *Pinus sylvestris* and *Larix decidua* are three species of Pinaceae that are of considerable economic and ecological importance in Central Europe. They cover together nearly two thirds of the about 10 million hectares of wood producing area in Germany. Similar to nearly all of the about 200 species of Pinaceae and many other gymnosperms they have more or less symmetric karyotypes consisting of 2n=24 morphologically rather similar chromosomes. According to Hizume (1988) the karyotype of genus *Pinus* is characterized by 1 or 2 pairs of medium-sized submetacentric chromosome pairs in addition to the larger metacentric ones; genus *Picea* has 8 large metacentric and 4 medium-sized chromosome pairs; genus *Larix* has 6 large metacentric and 6 medium-sized submetacentric pairs of chromosomes.

For *L. decidua*, idiograms have been published based on Chromomycin A3 (CMA) and DAPI banding (Hizume et al. 1993) and/or on chromosome length, arm length ratio and the visibility of secondary constrictions (Nkongolo and Klimaszewska 1995). For *Picea abies* (Terasmaa 1971, Hizume 1988) and *Pinus sylvestris* (Natarajan et al. 1961, Hizume 1988), idiograms or karyograms, respectively, have been published based on chromosome length, arm length ratio and visible secondary constrictions. However, these criteria were not sufficient for an unambiguous individualization of all of the chromosomes of these species due to similarity in size and arm length ratio, varying degrees of condensation of chromosomes between and within single metaphases and varying degrees of extension, and therefore visibility, of individual secondary constrictions.

Efforts to overcome this situation in *Larix* and *Pinus* species by Giemsa or fluorescence banding techniques resulted in banding patterns that either did not involve all of the critical chromosomes or were not informative enough (Borzan and Papes 1978, Drewry 1982, Hizume et al. 1993, Schubert unpublished). Attempts to distinguish chromosomes by means of hybridization signals after *in situ* hybridization with labelled rDNA have been reported for *Picea glauca* (Brown et al. 1993), *Pinus densiflora* and *P. thunbergii* (Hizume et al. 1992).

Here we report on our efforts to karyotype chromosomes of *Picea abies*, *Pinus sylvestris* and *Larix decidua* based on chromosomal localization of 18/25S and 5S rRNA genes by fluorescent *in situ* hybridization and on computer-aided chromosome analysis. By means of these techniques the chromosomes of the Norway spruce and some of the Scots pine and European larch karyotypes can be distinguished individually.

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Materials and methods

For chromosome preparation according to Schubert and Rieger (1994) root tips of 1-week-old seedlings, grown on wet filter paper, were used. After treatment for 15 h in 1% colchicine they were fixed overnight in 3:1 ethanol:acetic acid and digested in 1% cellulase (Onozuka R-10) and 1% pectinase (Sigma) for 1 h at 37°C. After squashing in 45% acetic acid, the slides were washed in ethanol for 5 min and air dried. Slides were immediately used for fluorescent *in situ* hybridization (FISH) or stored in glycerol at 4°C.

The plasmid VER 17 (Yakura and Tanifuji 1983, kindly provided by Prof. S. Tanifuji) containing 5.8S, parts of the 18S and most of 25S rRNA genes of *Vicia faba* cloned into pBR 325 was used as a NOR-specific hybridization probe. It was labelled either by Bio-16-dUTP (Boehringer Mannheim) or Digoxigenin-11-dUTP (Boehringer Mannheim) using a nick-translation kit (Amersham) according to manufacturer's instructions. A 5S rDNA-specific probe was amplified from genomic DNA of *V. faba* using primers designed according to the 5S rDNA sequence of *Glycine* (Gottlob-McHugh et al. 1990) and labelled by Bio-16-dUTP or Digoxigenin-11-dUTP via the polymerase chain reaction (PCR).

Heptameres of the *Arabidopsis*-type telomeric repeat (5′-TTTAGGG-3′) were labelled via PCR by Bio-16-dUTP (Boehringer Mannheim) according to Ijdo et al. (1991).

In situ hybridization was conducted as described by Fuchs and Schubert (1995) with one minor modification. The slides were heated for 15 min in 2× SSC at 94°C before proteinase K treatment. (This additional denaturation treatment improved the intensity of the hybridization signals on the chromosomes of all three species.) For sequential *in situ* hybridization, slides already hybridized with the rDNA probe were, after storage of the metaphase images, washed three times for 30–60 min in 4× SSC, 0.1% tween 20 and for 5 min in 2× SSC, dehydrated in an ethanol series and hybridized with a digoxigenin labelled 5S rDNA probe, or vice versa, according to Heslop-Harrison et al. (1992). Hybridization signals were evaluated using a ZEISS Axioskop with appropriate filter combinations. Images for each fluorochrome were taken separately, pseudocolored and merged as described in Fuchs et al. (1995). The complete images were printed on a Tektronix Phaser IISDX.

For karyotype analysis, chromosomes were captured by a cooled CCD camera and analyzed on a computer using the UNICHRO program (Ahne et al. 1989). The average of the chromosome length, chromosome arm ratio and position of hybridization signals was calculated for three complete metaphases with rDNA and two with 5S rDNA (including a reprobed one) of *P. abies*. The same was done for three metaphases, each after rDNA and 5S rDNA hybridization, of *L. decidua* and for two metaphases after rDNA and one after 5S rDNA hybridization of *P. sylvestris*.

Results and discussion

Fluorescent *in situ* hybridization with 18/25S rDNA-, 5S DNA- and telomere-specific probes

FISH with the 18/25S rRNA gene-specific probe yielded signals on 6 chromosome pairs of *Picea abies*, 3 chromosome pairs of *Larix decidua* and 7 chromosome pairs of *Pinus sylvestris* (Fig. 1a–c). This corresponds in the case of *L. decidua* to the number of secondary constrictions and CMA bands (Hizume et al. 1993; Nkongolo and Klimaszewska 1995). In the case of *P. abies*, Terasmaa (1971) observed only 5 pairs of satellite chromosomes, Hizume (1988) reported 11 satellite chromosomes but up to 12 interphase nucleoli, while Brown et al. (1993) found after FISH in the closely related species *P. glauca* up to 14 rDNA loci but only 5 pairs of chromosomes with a secondary constriction. In the case of *P. sylvestris*, Natarajan et al. (1961) observed 10, and Hizume (1988) 11, satellite chromosomes. Hizume et al. (1992) found 7 chromosome pairs with signals after hybridization with an rDNA probe in *Pinus densiflora* and 5 chromosome pairs with a strong and one with a weak hybridization signal in *P. thunbergii*. The latter species also revealed weak but clear signals at centromeric regions. The same observation was made in our experiments with *P. sylvestris*.

Results of FISH with 5S rDNA-specific probes have to our knowledge not yet been reported for Pinaceae species. *P. sylvestris* was revealed to have 4 loci, while *P. abies* and *L. decidua* have only 1 chromosome pair with 5S RNA gene clusters. In each case these loci occur on chromosomes that belong to the large metacentrics and that also harbor 18/25S rRNA genes.

Arabidopsis-type telomeric sequences (TTTAGGG, Richards and Ausubel 1988) have already been shown to label the chromosome ends and some interstitial positions of *Pinus* chromosomes (Fuchs et al. 1995). This could be confirmed, although the diffuse signal bands at noncentromeric interstitial positions were in most cases absent in our present experiments (data not shown). *P. abies* (Fig. 1d) and *L. decidua* chromosomes were observed to have hybridization signals at their termini. These signals were not informative for karyotyping and not detectable without preheating before proteinase K treatment (Fuchs et al. 1995).

Karyotyping by chromosome image analysis

Karyotype analysis of complete metaphase complements after FISH by means of the UNICHRO program was based on (1) the chromosome length; (2) the length ratio between the long and short arms (q/p); (3) the presence or absence of rDNA and 5S rDNA loci; (4) the position of these loci (a=the distance in percentage of chromosome arm length from the centromere).

The results obtained for *P. abies* are summarized in Table 1. rRNA genes were found on 6 chromosome pairs (II, III, IV, VI, VII and X). 5S rRNA genes were clustered at one locus on the long arm of chromosome II (Fig. 2a–c). Chromosome pair II is therefore characterized by loci for both genes. When chromosome length and the ratio between the chromosome arms were also taken into consideration the chromosome pairs of *P. abies* could be individualized.

For this purpose the chromosomes were divided into two groups: group I consisted of 6 chromosome pairs without rDNA/5S rDNA signals, while group II comprised 6 chromosome pairs with rDNA/5S rDNA signals. The latter was further divided into two subgroups. II/1, 3 chromosome pairs with rDNA/5S rDNA loci positioned within the short arm; II/2, 3 chromosome pairs with the rDNA loci located in the long chromosome arm.

The largest metacentric (q/p=1.10) pair of the group I chromosomes without a rDNA locus was clearly chromo-

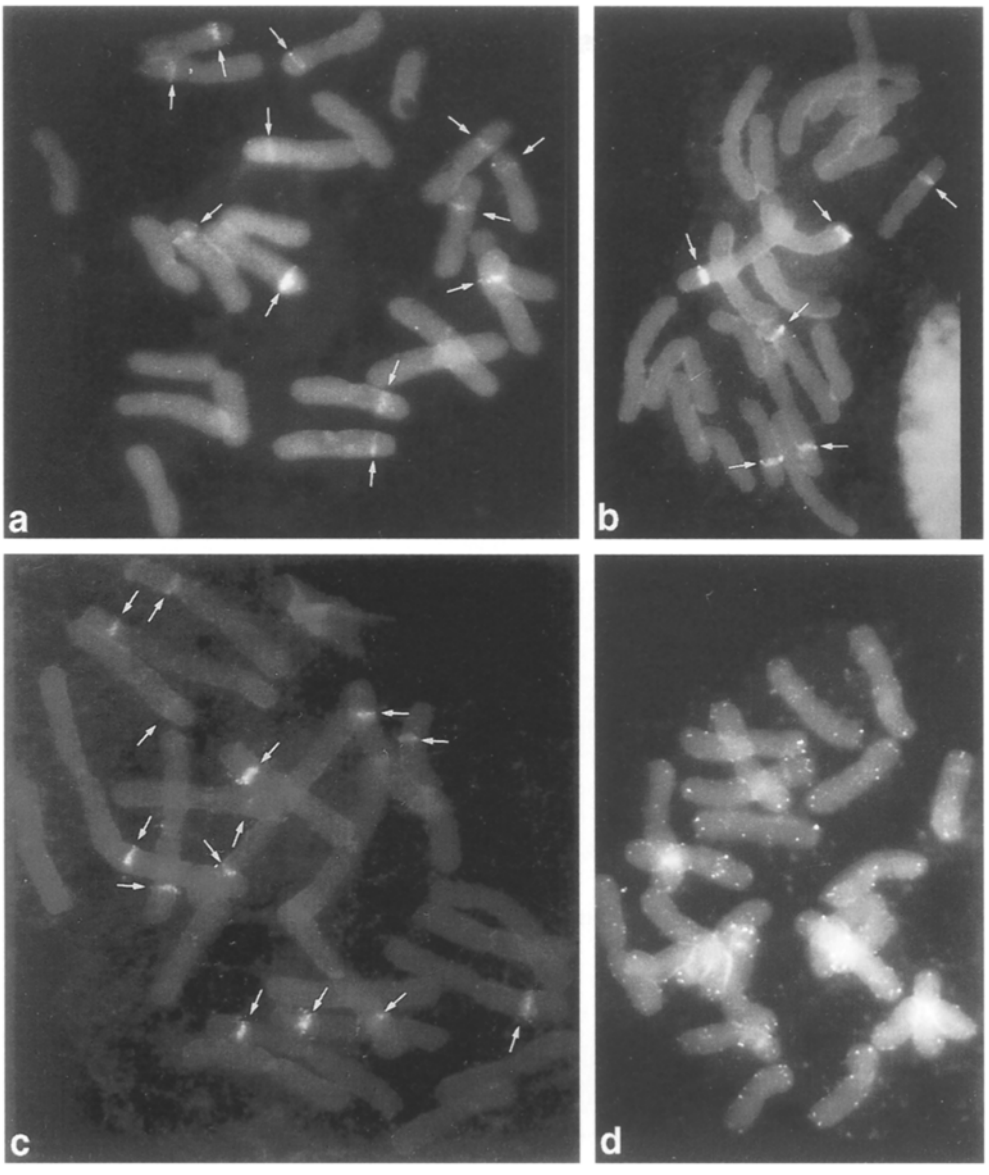


Fig. 1a–d Complete metaphases showing hybridization signals after FISH with biotinylated rDNA **a** *Picea abies*, **b** *Larix decidua*, **c** *Pinus sylvestris* or *Arabidopsis*-type telomeric repeats **d** *P. abies*. Note the weak labelling of some centromeres of *Pinus* chromosomes after FISH with the rDNA probe

some I, the second largest was determined to be chromosome V (q/p=1.17). Chromosomes VIII and IX are distinguishable on the basis of their centromere positions (q/p=1.21 versus 1.64). Chromosome XI (q/p=1.34) is similar in size and shape to the shortest pair (X) with an rDNA locus, while chromosome XII clearly represents the smallest and most asymmetric (q/p=1.86) pair of the karyotype. On the basis of its length, the largest metacentric pair (q/p = 1.12) of group II/1 with an rDNA locus in the short arm (a = 68.5%) was determined to be chromosome III, the second largest (q/p = 1.23; a = 66.5%) to be chromosome VI and the smallest one (q/p = 1.41; a = 59.7%) to be chromosome X. The largest metacentric (q/p = 1.11) pair of group II/2 with an rDNA locus on its long arm (a = 52.7%) – and the longest one with FISH signals – was determined to be chromosome II. It is additionally characterized by the 5S rDNA locus at its long arm closely proximal to the NOR (a = 45.0%). The next largest of this group is chromosome IV (q/p = 1.36; a = 46.7%). The shortest metacentric (q/p = 1.09) pair with an rDNA locus on its long arm is chromosome VII (a = 63.3%).

Among the group of the 8 large metacentric chromosomes (according to Hizume 1988) chromosome V could be mistaken for VIII and vice versa (both without rDNA loci) if the variation in degree of condensation is extremely high, since both have a similar arm ratio. The same is true for chromosomes III and VII (both containing an rDNA locus) since as a result of their rather symmetric shape the long and short arm may also be confused due to a certain degree of variability.

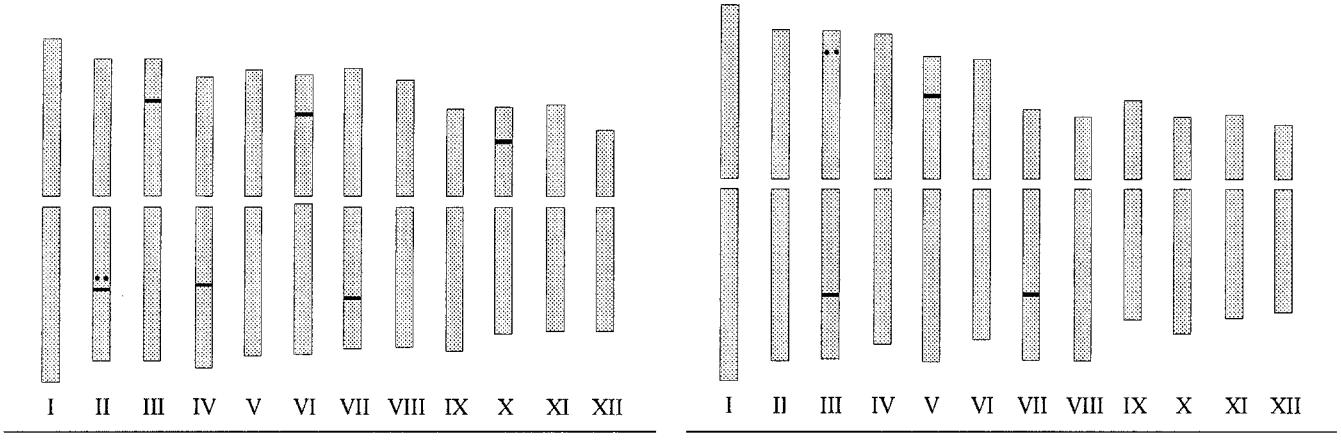
The same analysis was done for *L. decidua* (Table 2). This species revealed the lowest number of hybridization signals 6, with the NOR-specific probe. When chromosome length was taken into consideration, 2 of the 6 large

Table 1 Chromosomes of *Picea abies* arranged according to their relative length, arm length ratio (q/p) and position of rDNA/5S rDNA loci

Nr.	length SD	q/p SD	rDNA/ 5S rDNA	a (%) ± SD
I	10.67 ± 0.44	1.10 ± 0.07	–	–
II	9.30 ± 0.30	1.11 ± 0.12	q/q	52.7 ± 4.3/ 45.0 ± 0.8
III	9.27 ± 0.64	1.12 ± 0.11	p	68.5 ± 7.9
IV	8.96 ± 0.57	1.36 ± 0.17	q	46.7 ± 4.4
V	8.82 ± 0.47	1.17 ± 0.05	–	–
VI	8.72 ± 0.18	1.23 ± 0.15	p	66.5 ± 7.9
VII	8.64 ± 0.44	1.09 ± 0.14	q	63.3 ± 7.0
VIII	8.23 ± 0.31	1.21 ± 0.14	–	–
IX	7.43 ± 0.67	1.64 ± 0.23	–	–
X	6.96 ± 0.52	1.41 ± 0.23	p	59.7 ± 4.6
XI	6.94 ± 0.36	1.34 ± 0.08	–	–
XII	6.06 ± 0.40	1.86 ± 0.28	–	–

Table 2 Chromosomes of *Larix decidua* arranged according to their relative length, arm length ratio (q/p) and position of rDNA/5S rDNA loci

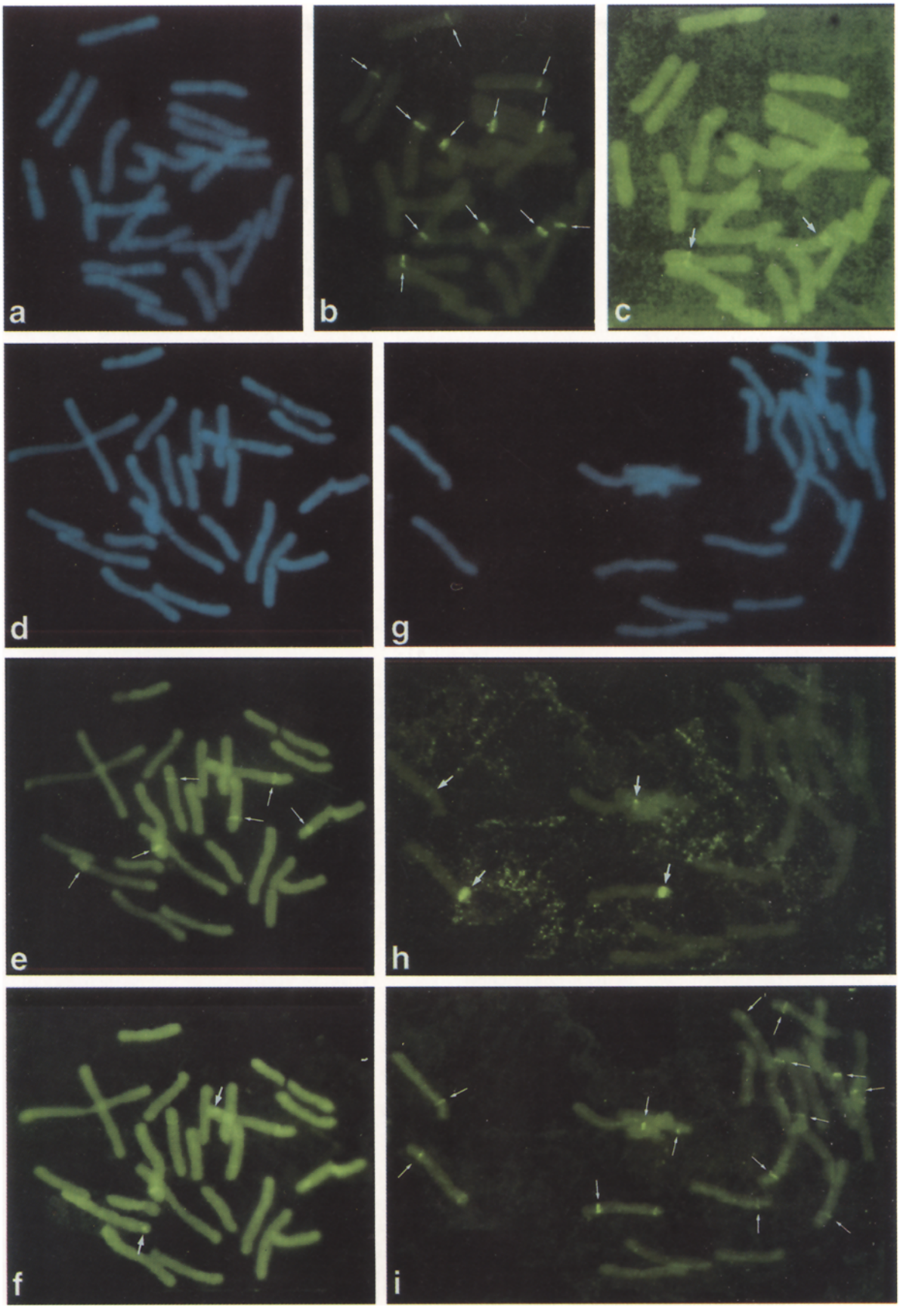
Nr.	length SD	q/p SD	rDNA/ 5S rDNA	a (%) ± SD
I	11.68 ± 1.00	1.10 ± 0.11	–	–
II	10.24 ± 0.54	1.15 ± 0.06	–	–
III	10.18 ± 0.60	1.14 ± 0.11	q/p	62.0 ± 7.0/ 82.7 ± 10.3
IV	9.63 ± 0.62	1.07 ± 0.15	–	–
V	9.38 ± 0.58	1.43 ± 0.25	p	67.2 ± 6.2
VI	8.71 ± 0.74	1.26 ± 0.17	–	–
VII	7.67 ± 0.39	2.51 ± 0.32	q	60.8 ± 6.4
VIII	7.44 ± 0.59	2.82 ± 0.50	–	–
IX	6.68 ± 0.58	1.67 ± 0.40	–	–
X	6.57 ± 0.36	2.38 ± 0.32	–	–
XI	6.14 ± 0.30	2.08 ± 0.21	–	–
XII	5.68 ± 0.41	2.30 ± 0.27	–	–



metacentric chromosome pairs, pair III (q/p=1.14; a=62.0% of the long arm) and pair V (q/p=1.43; a=67.2% of the short arm), as well as the longest of the group of the smaller, submetacentric chromosomes, pair VII (q/p=2.51; a=60.8% of the long arm), were determined to be NOR-bearing chromosomes. Chromosome pair III additionally revealed a signal with the 5S RNA gene-specific probe on its short arm (a = 82.7%) (Fig. 2d–f). In addition to these 3 chromosome pairs, chromosome pair XII can easily be identified since it is the shortest asymmetric pair of the karyotype. The chromosome pairs I, II, IV and VI of the group of large metacentric chromosomes on the one hand and pairs VIII, IX, X and XI of the group of smaller asymmetric chromosomes on the other are difficult to distinguish from each other due to their similar length and arm ratio. Even the bright fluorescent bands which are typical for all but 1 chromosome pair of *L. decidua* after DAPI staining (Hizume et al. 1993) are not of diagnostic value since the bands are positioned exclusively close to the centromeres. The fact that Hizume (1988), Hizume et al. (1993) and Nkongolo and Klimaszewska (1995) each determined different metacentric chromosomes and chromosome arms as carriers of secondary constrictions demonstrates that length measurements are too variable for unequivocal distinguishing all of the individual chromosome pairs of this species.

In the case of *Pinus sylvestris*, karyotyping proved to be even more difficult than in *Larix*. The largest chromosome pair (q/p=1.09) harbors loci for 18/25S RNA genes (a=54.0%) as well as for 5S RNA genes (a=34.7%) on the same chromosome, probably the short arm, and clearly represents chromosome I. There is another large metacentric pair (q/p=1.15) with rDNA and 5S DNA loci on opposite arms (a=60.5% and a=77.1%, respectively), but this chromosome, as most of the others, can not be enumerated exactly from its length (Fig. 2g–i). The third pair that can be easily discriminated is the smallest submetacentric pair XII; it hybridized with neither of the probes.

Fig. 2a–i Complete metaphases of *Picea abies* (**a–c**), *Larix decidua* (**d–f**) and *Pinus sylvestris* (**g–i**) after DAPI staining (**a**, **d**, **g**) and FISH with rDNA (**b**, **e**, **i**) and 5S rDNA probes (**c**, **f**, **h**). 5S rDNA loci are marked by *thick arrows*; rDNA loci by *thin arrows*. The rDNA probes were first hybridized to chromosomes of *Picea* and *Larix* followed by washing and reprobing of the same slides by 5S rDNA. For *Pinus* chromosomes the sequence of hybridization events was reversed.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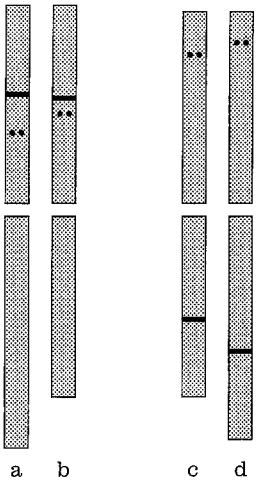

Fig. 3 Similar arrangements of rDNA and 5S rDNA loci on schematic chromosomes of *Pinus sylvestris* and *Picea abies* (**a**, **b**) and *P. sylvestris* and *Larix decidua* (**c**, **d**) suggesting their homoeologous relationship

The exact determination of homoeology between single chromosome pairs of different Pinaceae species is not yet feasible. Chromosome assigned linkage groups are not known as yet and complex DNA probes derived from microdissected chromosomes XII of *Picea abies* labelled *in situ* all of the chromosomes of *P. abies* and, to a lesser extent also all of the chromosomes of *Pinus sylvestris* (Fuchs et al. 1996). However, from the positions of rDNA and 5S DNA loci it might be speculated that chromosome I of *P. sylvestris* and chromosome II of *Picea abies*, on the one hand, and chromosome III of *L. decidua* and the chromosome of the Scots pine with both loci on opposite arms on the other, represent at least partially homoeologous linkage groups (Fig. 3).

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