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V. Zoldos · D. Papes · M. Cerbah · O. Panaud
V. Besendorfer · S. Siljak-Yakovlev

Molecular-cytogenetic studies of ribosomal genes and heterochromatin reveal conserved genome organization among 11 *Quercus* species

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Abstract Genomes of 11 *Quercus* species were characterized using cytogenetic (Giemsa C-banding, fluorochrome banding), molecular-cytogenetic (fluorescence *in situ* hybridization, FISH, to ribosomal genes) and molecular (dot-blot for ribosomal gene-copy number assessment) techniques. Ribosomal genes are the first DNA sequences to be physically mapped in oaks, and the copy number of the 18S-5.8S-26 S rRNA genes is estimated for the first time. Oak karyotypes were analysed on the basis of DAPI banding and FISH patterns; five marker chromosomes were found. In addition, chromosomal organization of ribosomal genes with respect to AT- and GC-differentiated heterochromatin was studied. Fluorochrome staining produced very similar CMA/DAPI banding patterns, and the position and number of ribosomal loci were identical for all the species studied. The 18S-5.8S-26 S rRNA genes in oak complements were represented by a major locus at the subterminal secondary constriction (SC) of the only subtelocentric chromosome pair and a minor locus at paracentromeric SC of one metacentric pair. The only 5 S rDNA locus was revealed at the paracentromeric region of the second largest metacentric pair. A striking karyotypic similarity, shown by both fluorochrome banding and FISH patterns, implies close genome relationships among oak species no matter their geographic origin (European or American) or their ecophysiology (deciduous or evergreens). Dot-blot analysis gave preliminary evidence for different copy numbers of 18S-5.8S-26 S rRNA genes in diploid genomes of *Q. cerris*, *Q. ilex*, *Q. petraea*, *Q. pubescens* and *Q. robur* (2700, 1300, 2200, 4000 and 2200 copies, respectively) that was correlated with the size polymorphism of the major locus.

Key words *Quercus* · Fluorochrome banding · FISH · Heterochromatin · Karyotype · 18S-5.8S-26 S and 5 S rRNA genes

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V. Zoldos · D. Papes · V. Besendorfer
Department of Molecular Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10 000 Zagreb, Croatia

V. Zoldos (✉) · M. Cerbah · O. Panaud · S. Siljak-Yakovlev
Laboratoire d'Evolution et Systématique, UPRESA – CNRS 8079, Université Paris XI, Bâtiment 360, 91405 Orsay Cedex, France
e-mail: vlatka.Zoldos@esv.u-psud.fr

Introduction

Oaks are one of the most important tree components of forest ecosystems of the Northern hemisphere, having therefore a great ecological and economical value. In addition, *Quercus* L. presents one of the world's richest genus among trees; more than 300 species (Schwartz 1964), with many varieties and natural hybrids, make this plant group taxonomically very complex. There are poor reproductive barriers between the species, resulting in frequent natural hybridization even between oaks that are very different both morphologically and physiologically. Some of those interfertile oaks can share the same ecological habitats but still remain distinct. Therefore, there has even been doubt whether the biological species concept, based on complete genetic isolation between species, is applicable to this genus (Burger 1975). The model of "ecological species" and "multispecies" has been proposed (Van Valen 1976). It supposes that, in this particular case of extensive hybridization and introgressive gene flow, selection for groups of co-adapted alleles in oaks' nuclear genomes maintains them as distinct species, characterized by correlated morphological and ecophysiological features.

Due to this situation, the systematic relationships and paths of evolution of the genus are still not well understood (Nixon 1993). Different approaches are taken in elucidating this problem; morphological (Spellenberg 1992; Bacilieri et al. 1995), enzyme (Guttman and Weigt 1989; Samuel et al. 1995) and DNA (Bodénes et al. 1997a, b) studies, as well as molecular analyses of chloroplast and/or nuclear ribosomal DNA (Bellarosa et al. 1990; Whittemore and Schaal 1991; Besendorfer et al. 1997; Samuel et al. 1998) have been conducted. Molecu-

lar-cytogenetics has not been performed on oaks so far, although techniques such as fluorochrome banding and fluorescence *in situ* hybridization (FISH) to ribosomal genes can be useful in studies of systematics and evolution (Mukai et al. 1991; Maluszynska and Heslop-Harrison 1993). The genome organization of constitutive heterochromatin and ribosomal genes have contributed in exploring karyo-evolutionary trends and/or phylogenetic relationships in quite a number of plant species (Godelle et al. 1993; Moscone et al. 1996; Cerbah et al. 1998a).

Given the ecological and economical importance of oak, numerous *Quercus* species have been subjected to extensive molecular ecology studies in order to protect and preserve their natural genetic resources (Hokanson et al. 1993; Bodénès et al. 1996; Le Corre et al. 1998). However, *Q. petraea* and *Q. robur*, the most important oaks in Europe with respect to the amplitude of their impact in forest ecosystems, have been the most thoroughly studied. Their genomes have been examined using a variety of DNA marker systems (Bodénès et al. 1996, 1997a, b; De Greef et al. 1998). In general, this is important for the identification of markers linked to economically important traits or to some resistance genes. Constructed genetic linkage maps are useful not only in breeding programmes, population genetics and studies of genetic structure and diversity, but also in studies of genome organization and evolution. We have learnt from personal contact with J.E. Carlson at Pennsylvania State University about projects including the development of oak databases and the sequencing of cDNA libraries. At this point, molecular-cytogenetics will be of great importance since once genetic maps are constructed there will be a need for associating the linkage groups with the chromosomes they represent, i.e. for physical mapping of any sequence or gene of interest.

At present, insufficient data is available on oak chromosomes. This is certainly due to their similar shape and small size and to difficulties in preparing good spreads. To date, oaks have been studied using classical cytogenetic methods, mostly to reveal their chromosome number. Just recently, modern cytogenetic techniques have been applied; Ohri and Ahuja (1990) constructed karyotypes of *Q. petraea, Q. robur* and *Q. rubra* with the aid of C-banding patterns, and Besendorfer et al. (1996) characterized heterochromatin by fluorochrome staining in *Q. petraea* and *Q. robur*. Nevertheless, chromosomes are difficult to pair unambiguously. Considering the wide interest that has been extended to this genus, deeper insight in oak cytogenetics appears to be an important task. Fluorochrome banding and FISH have already been shown to be valuable for the karyological characterization of species with small and similarly sized chromosomes (Maluszynska and Heslop-Harrison 1991; Schmidt et al. 1994). Here we report our efforts to identify oak chromosomes using cytological landmarks provided by these methods as well as to map repetitive DNA sequences as a first assay of FISH.

In genomes of higher eukaryotes, the 18S-5.8S-26 S (18S-26 S) and 5 S rRNA genes are present as multiple tandem repeats of coding units and intergenic spacers. They are located at one or more pairs of sites that can easily be visualized on chromosomes by FISH. In higher plants, these two ribosomal gene families are usually separated into distinct chromosomes. The number of rDNA chromosomal loci, as well as the number of rDNA repeats within the genome, mostly vary between related species, thus revealing their relationships (Maluszynska and Heslop-Harrison 1993; Cerbah et al. 1998a).

The investigation presented here involved: (1) a comparative study of the genome organization of ribosomal genes and heterochromatin in 11 *Quercus* species, belonging to four subgenera and originating from both Europe and North America, in order to learn about their taxonomic relationships, (2) physical markers for chromosomal identification, and (3) an estimation of 18S-26 S rRNA gene copy numbers in genomes of different species.

Materials and methods

Plant material and chromosome preparation

The taxonomy of 11 *Quercus* species and the localities of their collection are described in Table 1. The diploid chromosome number was 2n = 2x = 24 for all oaks investigated. Root tips were obtained from seedlings germinated from acorns on moist cotton in petri dishes at room temperature and then fixed in ethanol:acetic acid (3:1) after treatement with 2 m*M* 8-hydroxyquinoline at 16–18°C for 4 h. Chromosome preparations for fluorochrome banding and FISH were done using the air-drying technique of Geber and Schweizer (1987) with modifications. Briefly: fixed root tips were incubated in an enzymatic mixture [2% (w/v) cellulase, 1.5% macerozyme, 0.3% pectolyase Y23, 0.03% EDTA in 2×SSC, pH 4.2] at 37°C for 1.30–3 h (depending on the species and/or thickness of the root tips), the lysate of 1 root tip was then centrifuged at 800 *g* for 5 min, the supernatant was removed and the cellular mass was washed twice in citrate buffer, then twice in fresh ice-cold fixative by centrifugation. Finally, the pellet was resuspended in an appropriate volume of fixative, and about 40 µl was dropped onto a clean slide and air-dried.

Orcein staining, Giemsa C-banding and fluorochrome banding

Root tips were hydrolized 15 min in 1 *M* HCl at 60°C prior to staining in 2% lacto-propionic orcein for few hours to overnight in order to achieve better colouration. They were then macerated in an enzymatic mixture for 15–20 min and squashed in 45% acetic acid. Giemsa C-banding was performed according to Ohri and Ahuja (1990) with minor modifications: chromosomes were denatured in 6% barium hydroxide solution at 50°C for 6 min. For colouration, 2% Giemsa in Sorensens' buffer was used. Chromosomes were sequentially stained with Chromomycin A3 (CMA) and 4,6-diamidino-2-phenylindole (DAPI) following the protocol of Schweizer (1976). CMA (Sigma) was used at a concentration of 0.125 mg/ml and DAPI (Sigma) at 0.2 µg/ml. CMA and DAPI colourations were performed 13 and 10 min, respectively. Some slides were stained sequentially with CMA after FISH.

Fluorescence *in situ* hybridization

Two ribosomal DNA probes were used for double-target FISH. The 18S-26 S rDNA probe, isolated from *Arabidopsis thaliana*, was a clone of a 4-kb *Eco*RI fragment containing a part of the 18 S and the entire 5.8 S and 26 S coding region together with non-transcribed spacers (provided by Dr. D. Schweizer). Clone

Table 1 *Quercus* species studied, taxonomy according to Schwartz (1964) and the localities of the populations' collection. [**C** subgen. *Cerris* (Spach.) Örsted., **S** subgen. *Sclerophyllodrys* O., **E** subgen. *Erythrobalanus* (Spach) Örsted., **Q** subgen. *Quercus* (subgen. *Lepidobalanus* (Endl.)]

Species	Taxonomy	Provenance	Collector
Q. cerris L.	C	Croatia, Dakovo (Slavonia)	J. Franjic, T. Littvay, 1995–96
Q. coccifera L. [a]	S	France, Corniche de Cassis (Provence)	S. Siljak-Yakovlev, 1996
Q. ilex L. [a]	S	France, Ile de Porquerole (Provence)	J. P. Henry, 1996
Q. michauxii Nutt.		USA, Alachua Country (Florida)	J. M. Briantais, J. Cavender, 1995
Q. palustris Muenchh.	E	USA, Alachua Country (Florida)	J. M. Briantais, J. Cavender, 1995
Q. petraea (Matt.) Lieb.	Q	France, Forest of Dourdan (Parisian region)	V. Zoldos, O. Panaud, 1996
		Croatia, mountains of Moslavina	J. Franjic, T. Littvay, 1995–96
Q. pubescens Willd.	Q	Croatia, Motovun (Istria)	J. Rosa, 1996
Q. robur L.	Q	France, campus of Orsay University (Parisian region)	V. Zoldos, O. Panaud, 1996
		Croatia, Bjelovar (Slavonia)	J. Franjic, T. Littvay, 1995–96
Q. rubra L.	E	France, campus of Orsay University (Parisian region)	J. P. Henry, 1997
Q. suber L.	C	France, Lavandou (Provence)	J. P. Henry, 1996
Q. virginiana Ten. [a]	Q	USA, Alachua Country (Florida)	J. M. Briantais, J. Cavender, 1995

[a] Evergreen species

pTa794, which contained complete 410-bp *Bam*HI fragment of the 5 S rRNA gene and spacer region of wheat (Gerlach and Dyer 1980), was used as the 5 S rDNA probe. The 18S-26 S rDNA probe was labelled with digoxigenin-11-dUTP (Boeringher-Mannheim) and pTa794 with Fluoro-Red-dUTP (Amersham) using the polymerase chain reaction (PCR). For *in situ* hybridization, slides were pretreated with 100 µg/ml of RNase in 2×SSC at 37 °C for 1 h and subsequently incubated in pepsin (Sigma, 5 µg/ml in 0.01 *M* HCl) at 37 °C for 15 min. Slides were then dehydrated in a graded ethanol series and air-dried. DNA:DNA hybridization, stringent washes and detection was carried out as described by Heslop-Harrison et al. (1991) with slight modifications. The probes were mixed at a final concentration of 1.2–2.0 ng/µl in a solution of 50% (v/v) formamide, 10% (w/v) dextran-sulphate, 0.1% (w/v) sodium dodecyl-sulphate, 250 µg/ml salmon sperm and 2×SSC, immediately prior to *in situ* hybridization. Chromosomal DNA and the probes were denatured simultaneously at 80 C for 3 min, and then put at 37 °C to allow their hybridization overnight. Preparations were counter-stained with 2 µg/ml DAPI and then mounted in anti-fade solution (AF1 Citifluor). Observations were done using a Zeiss Axiophoton epifluorescence microscope with different combinations of Zeiss excitation and emission filters. Hybridization signals were analysed using the highly sensitive CCD camera (Prinston) and image analyzer. The chromosomes' superimposed images were a three-coloured contrast manipulated but not otherwise processed.

DNA extraction

Total genomic DNA was extracted from the fresh or frozen leaves according to Doyle and Doyle (1987) with slight modifications. The extraction buffer was composed of 0.1 *M* Tris HCl, pH 8, 1.25 *M* , NaCl, 0.02 *M* EDTA, 4% MATAB, and 0.1% β-mercaptoethanol. The DNA was treated with RNase.

Estimation of rDNA copy numbers

Copy numbers of the rDNA genes in oak species were estimated by making several dilutions of the 18S-26 S rDNA probe (the same one as used for FISH), corresponding to various copy numbers, as well as by making several dilutions of the oak total genomic DNA, dot-blotting these on positively charged nylon membranes (Hybond-N+, Amersham) using a Dot-Blot apparatus and then alkali-fixing them following the manufacturer's recommendations. Membranes were hybridized with the 18S-26 S rDNA probe labelled with digoxigenin-11-dUTP (Boehringer Mannheim), and washed twice with increasing stringency (maximum 0.5×SSC at 65°C); and chemiluminescent signals were detected using protocols described by Panaud et al. (1993). Since the estimation of the rDNA copy number in tomato has been reported by Ganal et al. (1988) using a similar approach, total genomic tomato DNA was included in the experiment as a control.

Results

Orcein staining and Giemsa C-banding

Orcein staining revealed that complements of *Q. petraea* and *Q. robur* consisted of metacentric to submetacentric chromosomes, with the exception of one subtelocentric pair (Fig. 1a). However, weak morphological differentiation prevented karyotyping. The subtelocentric pair and one (sub)metacentric pair bore nucleus organizes regions (NORs). The subtelocentric satellite chromosome pair showed a high fragility of its subterminal secondary constriction (SC). Satellites were often detached and seen in the cells as independent chromatin bodies (Fig. 1c). Paracentromeric SCs of the submetacentric pair were mostly difficult to observe due to chromatin condensation.

Giemsa C-banding revealed centromeric C-bands on almost all chromosomes (Fig. 1b). The most prominent ones appeared on two pairs (Fig. 1b, arrowheads); one pair of C-bands corresponded to the paracentromeric SCs and another one was positioned at the paracentromeric region of the second largest pair of chromosomes. On some spreads, hazy thin intercalary C-bands were observed, but banding resolution was not enough good to permit using these as markers in karyotyping. Two strongly coloured heterochromatic blocks were present terminally on two chromosomes, and they corresponded to the position of the (sub)terminal NORs (Fig. 1b, arrow). Alternatively, one or two entirely heterochromatic satellites were observed to be detached (Fig. 1c, arrows). C-banding was carried out on *Q. cerris*, *Q. petraea*, *Q. pubescens* and *Q. robur*. Satisfactory good C-banded metaphases of other species were not achieved for com-

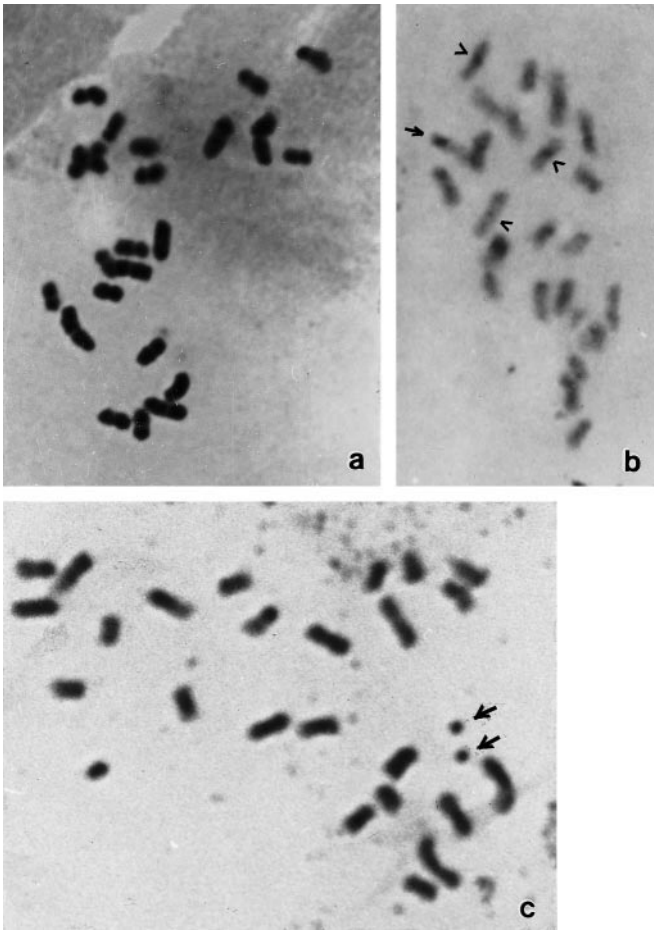


Fig. 1a Orcein-stained and Giemsa C-banded chromosomes of *Q. petraea*. Note the most prominent centromeric C-bands (**1b**, *arrowheads*), telomeric C-blocks (**1b**, arrow) or heterochromatic satellites, (**1c,** *arrows*). Magnification ×1300

parative analyses. We also noticed SCs on Giemsa-, CMA- or DAPI-stained chromosomes of the largest pair (Fig. 2 i,j, arrows), but these SCs did not contain ribosomal genes as revealed after FISH (see following text).

Fluorochrome banding

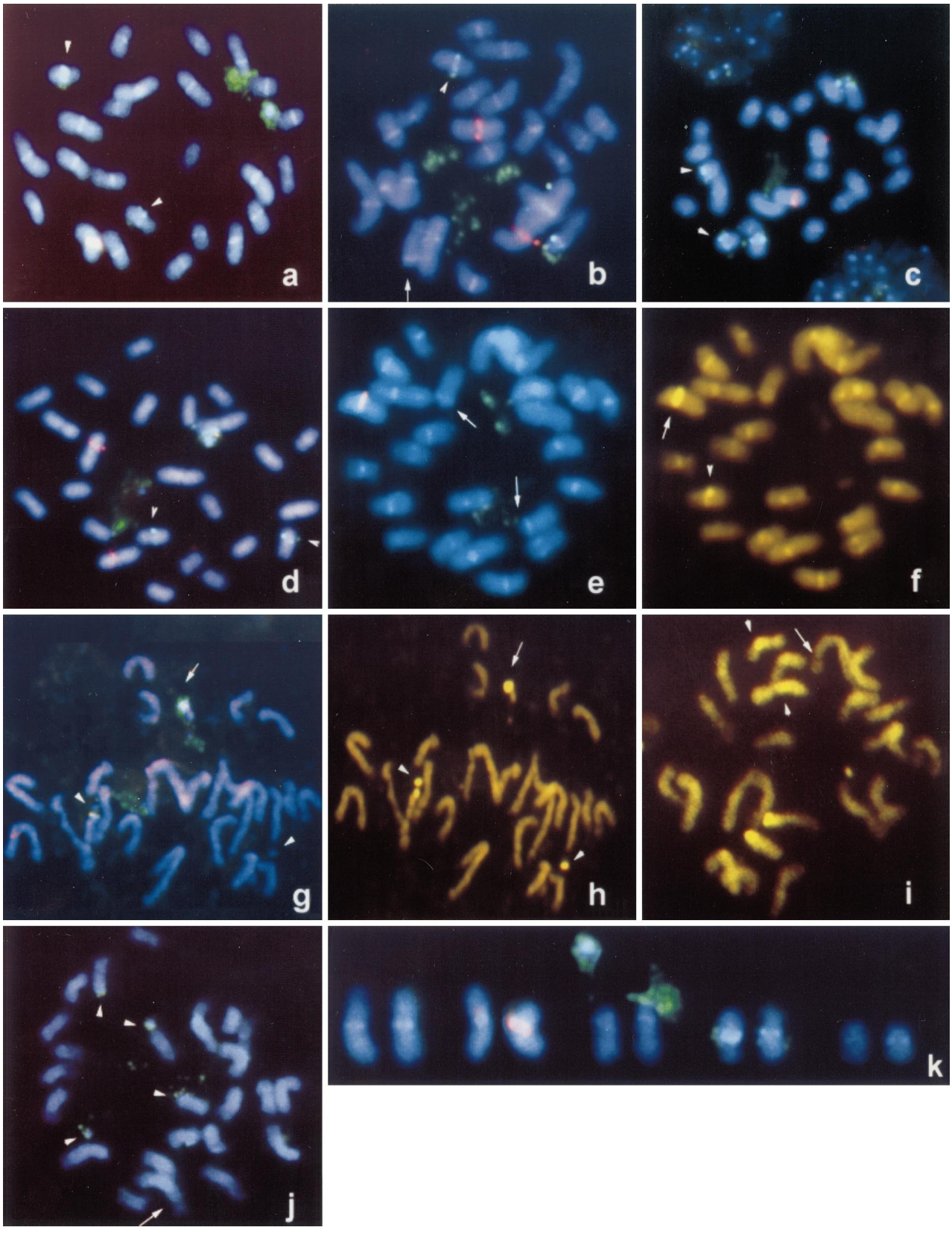
DAPI staining initially performed according to Schweizer (1976) produced weak banding. Both, DAPI and CMA patterns were improved, but not changed, if fluorochrome stainings were applied following FISH. Denaturation of chromosomal DNA prior to *in situ* hybridization probably increased the access of the dyes to AT- or GC-rich DNA, as has already been observed in some plant species (Maluszynska and Heslop-Harrison 1993). Accordingly, DAPI staining resulted in prominent bands at the centromeric region of all chromosomes in *Q. cerris*, *Q. michauxii*, *Q. petraea*, *Q. pubescens*, *Q. robur* and *Q. rubra* (Fig. 2a,b,c,e). No other, interstitial or terminal, DAPI bands were observed. Uniform DAPI staining lacking specific bands appeared on chromosomes of *Q. coccifera*, *Q. ilex*, *Q. palustris*, *Q. suber* and *Q. virginiana* (Fig. 2 g,j).

Fluorochrome staining with CMA revealed bands on all chromosomes at a juxtaposition with those produced with DAPI (compare Fig. 2e and f). Two chromosomes of the different pairs displayed the most prominent CMA bands (Fig. 2f,i, arrowheads/arrow in f), which corresponded to the FISH signals (see below). Large C-banded terminal blocks and/or satellites were the most CMA-fluorescent (Fig. 2h,i) and the only DAPI-negative regions (compare Fig. 2g and h, arrowheads). No other CMA marker bands appeared on any of the chromosomes. This basic CMA pattern was observed for all of the species investigated. However, some differences were noticed in *Q. cocciferea*, *Q. ilex*, *Q. suber*, *Q. palustris* and *Q. virginiana*. Only the most prominent CMA bands and terminal CMA dots were evident in their metaphases (Fig. 2h, i, arrowheads), and a reduced number of CMA chromocentres was present in their interphase nuclei compared to those of other species studied.

Physical mapping of ribosomal RNA genes

The number and position of rDNA loci were identical for all 11 species studied; four FITC-green and two Fluoro-Red hybridization signals, corresponding to four sites of 18S-26 S and two sites of 5 S rRNA genes, respectively, appeared on DAPI-blue metaphase chromosomes (Fig. 2a-e,g,j). Two 18S-26 S rDNA loci differed in signal size and intensity; the major locus on the subtelocentric satellite chromosome pair was named NOR-1 and the much smaller minor locus at the paracentromeric SC of one medium-sized metacentric pair was designated as NOR-2 (Fig. 2a-e, arrowheads). The size and intensity of the hybridization signals also differed between homologous sites of NOR-2, or one site of the locus was not always detectable (Fig. 2e). The site displaying the stronger sig-

Fig. 2a-e,g Double-target FISH to chromosomes of *Q. robur* **(a)**, *Q. cerris* **(b)**, *Q. petraea* **(c)**, *Q. michauxii* **(d)**, *Q. pubescens* **(e)** and *Q. suber* **(g)**. Fluoro-*Red* signals correspond to the only 5 S rDNA locus and FITC-*green* signals to 18S-26 S rRNA genes; *arrowheads* indicate the minor 18S-26 S rDNA paracentromeric locus; dispersed *green* particles indicate decondensed major 18S-26 S terminal sites (*arrows* in **e** indicate a subtelocentric pair missing the FISH signal due to the complete decondensation); *arrowheads* in **g** show DAPI-negative satellites that are not labelled with the 18S-26 S rDNA probe but are CMA-positive (compare with **h,** arrowheads). **f, h, i** CMA-banded metaphases of *Q. pubescens*, *Q. suber* and *Q. coccifera*, respectively (compare with **e** and **g**); note the difference of the banding patterns in terms of number of CMA bands between *Q. pubescens* and two evergreen species (*arrow* in **f** indicates the most prominent CMA band that corresponds to the 5 S rDNA site). **j** Single-target FISH with 18S-26 S rDNA probe to chromosomes of *Q. ilex*; *arrowheads* indicate the presence of hybridization signal on just half of the satellites and terminally on the corresponding NOR-bearing chromosomes (*arrows* in **i** and **j** indicate the SC on the largest chromosome). **k** Five marker elements in oak complements, three of them carrying FISH landmarks. Magnification ×1600 in **a**, **c**, **d**, **g**, **h** and **j**; and ×2300 in **b**, **e**, **f**, **i** and **k**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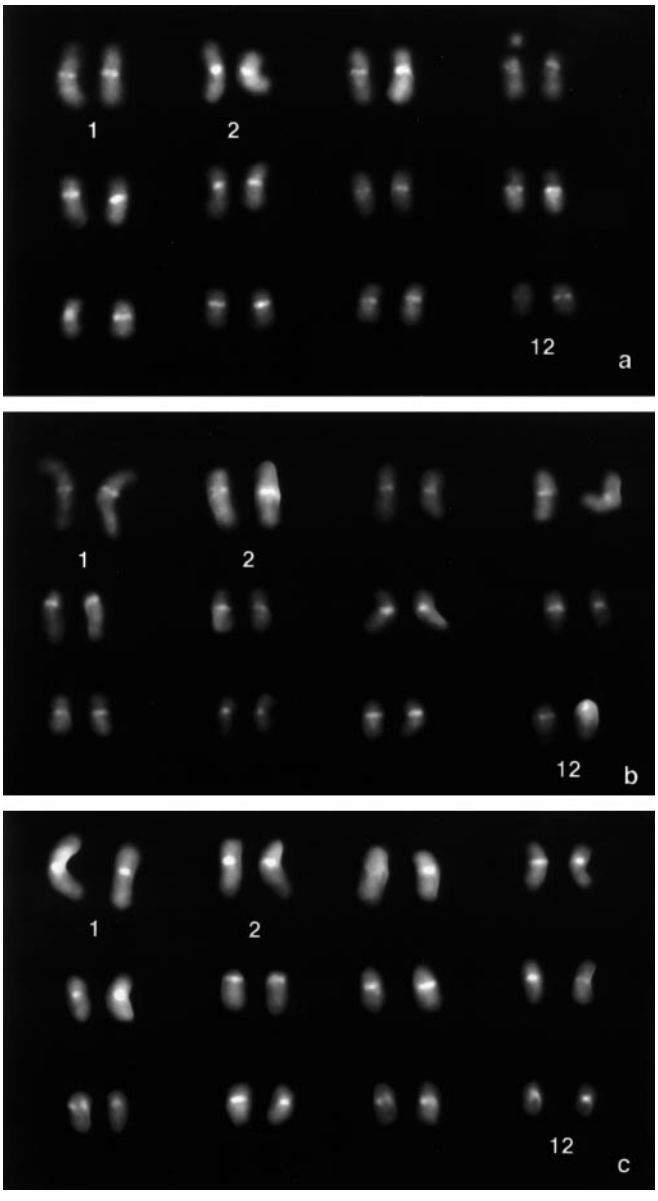

Fig. 3 a-c Chromosomes of *Q. robur* **(a)**, *Q. cerris* **(b)** and *Q. pubescens* **(c)** paired on the basis of their relative length and arm ratio, where DAPI bands were taken as the position of the centromeres, and using FISH markers (see corresponding metaphases in Fig. **2a**, **b** and **e**). Three pairs are numbered as 1, 2 and 12. Magnification ×2300

nal corresponded to one of the most prominent C- and CMA bands, as revealed after sequential staining (compare Fig. 3e and f).

The NOR-1 locus showed some properties that made it dynamic, hence disturbing the uniformity of the FISH pattern. This locus was distinctive in:

1) interspecific differences in the distribution of ribosomal genes at proximal and distal part of SCs and/or on satellites, i.e. in *Q. ilex*, *Q. suber*, *Q. virginiana* and *Q. palustris* signals were present subterminally on chromosomes or just on part of the satellites (Fig. 2g,j), while in all other species they were found terminally on chromosomes or on entire satellites (Fig. 2a,b).
2) unequal decondensation of homologous sites. The rDNA of both sites was sometimes completely decondensed so that NOR-bearing chromosomes were not marked at all (indicated in Fig. 2e by arrows).
3) perfect correspondence with CMA-marked areas. In other words, the entire region that displayed CMA fluorescence hybridized with the 18S-26 S probe (compare Fig. 2g and h, arrows). However, in a few cases, small satellites, which were CMA-positive, did not display the hybridization signal (compare Fig. 2g and h, arrowheads).
4) size dimorphism of the homologous sites that was not inevitably connected with rDNA decondensation.

The only 5 S rDNA locus was positioned at the paracentromeric region of the second largest (sub)metacentric pair (Fig. 2a-e,g). Dimorphism of the two sites was prominent in some metaphases (Fig. 2a,c,e,g), and was related to the polymorphism of C- and CMA-positive bands at the same position (Fig. 2f).

Karyotypes of oak species

The CMA and DAPI banding patterns were not of diagnostic value except for the position of the centromeres. On the other hand, FISH specifically labelled six chromosomes. Five pairs were recognizable in all oak complements, so they could be regarded as marker elements (Fig. 2k):

1) the biggest metacentric pair;
2) the second largest (sub)metacentric pair bearing paracentromeric 5 S rDNA locus;
3) the medium-sized subtelocentric pair bearing (sub)terminal NOR-1 locus;
4) the medium-sized metacentric pair marked with paracentromeric NOR-2 locus;
5) the smallest pair recognizable on the basis of its size.

All other chromosomes were difficult to distinguish because of continuously decreasing sizes, homogenous morphology and lack of cytological markers. In addition, they showed differential condensation throughout pro- and metaphase stages, so that the exact boundaries between chromosomal types were hard to establish. However, we tried to pair them on the basis of total length and arm ratio, where centromeres were taken as the position of the centromeric DAPI bands (measurements not presented). Karyotypes of *Q. petraea*, *Q. cerris* and *Q. pubescens* given in Fig. 3 were produced from at least five metaphases of each species. Prominent heterology of the arm ratios was noted for the four longest chromosomes, but these were paired unambiguously with the aid of the marker 5 S rDNA locus. The main NOR-bearing pair was dimorphic for both arms, also for the short one due to the size polymorphism of the satellite region. Therefore, the homologues varied in total length between each other and in different metaphases. Since the length

measurements were too variable for unequivocal chromosome ranking, just three pairs were numbered in karyograms; the largest pair (pair 1), the pair bearing the 5 S rDNA locus (pair 2) and the smallest one (pair 12).

Estimation of 18S-26 S rRNA gene copy number

Using our dot-blot hybridization experiments, we estimated the copy numbers of rDNA as 2300 in the tomato genome, which was similar to the results previously published (Ganal et al. 1988). Based on a genome size of 1.8–2.0 pg/2 C for *Quercus* genus (Zoldos et al. 1998), our reconstruction experiments led to the following estimations of rDNA copy numbers: 1300 for *Q. ilex*, 2200 for *Q. petraea* and *Q. robur*, 2700 for *Q. cerris* and 4000 for *Q. pubescens*.

Discussion

Oak karyotypes

The molecular cytogenetic analysis of 11 *Quercus* species showed that basic chromosome features remain unaltered within the genus. Oak karyotypes consist of sub- to metacentric chromosomes and only one subtelocentric satellite pair. The two biggest pairs clumped out from the group of the middle-sized chromosomes of similar shape. According to Stebbins' (1971) classification, karyotypes are moderately asymetrical. Karyograms constructed with the aid of centromeric DAPI bands and FISH markers in general agree with karyograms that have already been provided for some oak species. Ohri and Ahuja (1990) reported six metacentric, five submetacentric and one subtelocentric pair and SCs/satellites only on the (sub)metacentric pair II in *Q. petraea*, *Q. robur* and *Q. rubra*, while Medjedovic (1983) found different number ratios of metacentrics and submetacentrics and positional variation of SCs from population to population of *Q. petraea*. Small discrepancies of chromosomal types in karyotypes of the same species, established by previous authors and herein, could be due to unequal chromatin condensation and/or different chromosome preparation. According to these earlier reports, it seems that some oak populations possess a variable number/position of SCs. We found two pairs that constantly bore SCs associated with ribosomal genes, i.e. NORs. The SC observed on the biggest chromosome pair could correspond to the SC that observed by Ohri and Ahuja (1990) on the pair II. However, this SC did not contain ribosomal genes as revealed by FISH. Fragility of the subterminal SC resulted in the frequent appearance of polymorphic satellites in oak metaphases. The characteristic shape of the satellites – i.e. spherical compact chromatin bodies, usually not connected with any visible extension with the "mother" chromosomes – could lead to misidentification with B-fragments or B-chromosomes (Medjedovic 1983, Ohri and Ahuja 1990).

New cytological landmarks are needed to distinguish between chromosomes of the group of middle-sized (sub)metacentrics. However, considerable progress in oak cytogenetics has been made. This includes:

1) clean chromosome spreads obtained by protoplast liberation. This was achieved using an enzymatic mixture found to be the best for maceration of oak root-tip meristem, whose cells contain a lot of cellular inclusions, as well as using pepsin treatment to minimize cytoplasm residue;
2) identification of marker chromosomes by means of double-target FISH in combination with DAPI counter-staining;
3) the use of the highly sensitive CCD camera in combination with the computer software for easier analysis of small oak chromosomes.

Structural organization of heterochromatin and ribosomal genes

The largest fraction of constitutive heterochromatin in the oak genome was associated with NORs and was exclusively GC differentiated, similar to the situation in the majority of plant and animal species. Conversely, it seemed that the rest of the heterochromatin, located close to the centromeres of all chromosomes, consisted of both GC- and AT-rich intermingled repetitive sequences. Such an organization has been observed in *Medicago truncatula* cv 'Jemalong' (Cerbah et al. 1998b), on some chromosomes of *Pinus elliottii* (Doudrick et al. 1995) and *Reichardia gaditana* (Siljak-Yakovlev et al. 1998).

Perfect correlation of CMA and FISH marked areas on oak NOR-bearing chromosomes indicated the co-localization of GC-differentiated heterochromatin and ribosomal genes and raise the question concerning the organization of these sequences relative to each other. In general, two different explanations have been given: the ribosomal tandem repeats could either be interspersed within heterochromatin or they would themselves constitute highly repetitive sequences of heterochromatin. The heterochromatic location of tandemly repeated genes, comparable to that found for the rRNA genes in oaks, have been widely reported, thereby providing experimental support for the first hypothesis (Pardue et al. 1973; Fitch et al. 1990; Mukai et al. 1991; Pendás et al. 1993, 1994; Abuin et al. 1996). Associations of genes with highly repetitive sequences would increase the opportunity for unequal exchanges and gene duplications, therefore increasing their copy number. Size dimorphism of the hybridization signals, CMA/DAPI bands and C-bands of the same position at homologous sites for both 18S-26 S rDNA and 5 S rDNA loci in oaks could be explained by such events. It has been shown in some cases that the size difference in rDNA C-bands is correlated with an equivalent change in the amount in rDNA (Brettel et al. 1986; Mukai et al. 1991).

FISH is considered to be a semi-quantitative technique (Maluszynska and Heslop-Harrison 1993), i.e. sig-

nal size and strength reflect a copy number of these genes. Most of the oak 18S-26 S rDNA repeats were localized at the major locus (NOR-1) as revealed by a very strong FISH signal, and the rest was organized as a much smaller cluster at the minor locus (NOR-2). The NOR-1 locus showed signal size variability between homologous sites, as well as between metaphases of different species, while the size difference ratio of the NOR-2 sites remained constant. Therefore, it seems that preliminary evidence for different copy numbers of 18S-26 S rRNA genes in genomes of some *Quercus* species is correlated to the size polymorphism of the major locus. It should be noticed that intraspecific variability for a number of rRNA repeats was not examined, although size differences in the NOR-1 *in situ* hybridization signals were observed between some individuals.

Relationships of species

The absence of chromosome differentiation between different oaks studied herein lead to the conclusion that they possess one common karyotype. In addition, according to double-target FISH applied to 11 species of different taxonomic groups and distinct geographic origin, it seems that the position and number of 18S-26 S and 5 S rDNA loci are probably highly conserved within the genus. The basic pattern of fluorochrome banding, with respect to chromosomal position of the bands, was evident, although the reduced number of CMA/DAPI bands and chromocentres in the case of *Q. suber*, *Q. ilex*, *Q. coccifera* and *Q. virginiana* could present the quantitative differences in heterochromatin between species. However, these differences have not been detected by estimation of GC percentage in their genomes, or the differences are too small to be revealed by flow cytometry (Zoldos et al. 1998). Estimation of nuclear DNA content (Ohri and Ahuja 1990; Favre et al. 1996; Zoldos et al. 1998), C-banding (Ohri and Ahuja 1990), fluorochrome banding and the FISH patterns of 11 oaks have revealed a striking interspecific similarity at the levels of genome size and organization. Despite such genomic similarity, which probably led to the loss of reproductive barriers and therefore to an extensive natural hybridization (Rushton 1993), *Quercus* species remained distinct, both morphologically and ecophysiologically. Thus, the present work represents a new contribution to the conception which considers *Quercus* species as dominant groups of individuals characterized by co-adapted alleles and correlated phenotypic features (Van Valen 1976). It is curious that the pattern of chromosomal organization of heterochromatin and ribosomal genes has remained identical for geographically distinct species, despite the fact that sequences making up heterochromatin are the ones that evolve the most rapidly in genomes and that ribosomal genes have been proven to be excellent markers in systematic and evolution (Cerbah et al. 1998a). However, it seems important to have an insight into the chromosomes of Asian species.

Conclusions and prospects

The present work reports for the first time the physical mapping of any of the DNA sequences to oak chromosomes. FISH application to angiosperm forest trees is rare; to our knowledge ribosomal genes have only been labelled in interphase nuclei of some *Populus* species (Prado et al. 1996). Considerable progress in oak molecular-cytogenetics has been made. The use of the air-drying technique has provided good metaphase spreads with a minimum cytoplasm; this has resulted in an extremely low background and a high reproducibility of FISH signals. In addition, two rRNA gene classes simultaneously mapped on DAPI-banded chromosomes provided marker elements in oak complements. The present results show that a combination of these techniques is valuable in the physical mapping of repeated DNA sequences in oaks and, by enhancement of FISH resolution, it gives the opportunity for low- and/or single-copy mapping. Given the broad interest being extended to this important woody genus, an integrated molecular and cytogenetic model of oak will soon be required. On the other hand, FISH to ribosomal genes showed conserved genome organization within the genus and did not yield any arguments about taxonomic relationships. Isolation of major classes of oak repetitive DNA and their physical mapping through FISH application is currently in progress. This would permit a more detailed analysis of genome organization and evolution in *Quercus*.

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