Tree Genetics & Genomes (2011) 7:1113–1122
DOI 10.1007/s11295-011-0399-x

ORIGINAL PAPER

Evolution of rDNA FISH patterns in the Fagaceae

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Received: 20 July 2010 / Revised: 15 January 2011 / Accepted: 11 May 2011 / Published online: 4 June 2011

Abstract The Fagaceae is one of the most important plant families in European forest ecosystems, and it includes several genera distributed in the Northern hemisphere. In this work we studied the genome organization and evolution within the family, by karyotyping and physically mapping rDNA in ten European and Asian species of the genera *Fagus*, *Quercus*, and *Castanea*. All of the species studied had a chromosome number of 2n=2*x*=24, except for the first report of a single individual of *Quercus suber* which proved to be triploid (2n=3*x*=36). The rDNA physical mapping revealed several patterns: the dominant one is present in European and Asian *Quercus* subgenus *Quercus*, and in *Castanea sativa* and *Castanea crenata*, consisting of two 18S–25S rDNA loci (one subterminal major and one pericentromeric minor) and one 5S rDNA pericentromeric locus. In *Fagus sylvatica* and in *Quercus sessilifolia*, different patterns were observed: four terminal 18S–25S rDNA loci and two 5S rDNA pericentromeric loci in the former, and five 18S–25S rDNA loci (three terminal and two intercalary) and one 5S rDNA pericentromeric locus in the latter. In *Castanea mollissima* a distinct rDNA distribution pattern with two intercalary 18S–25S rDNA loci and two 5S rDNA was found. These findings suggest rDNA loci restructuring during *Castanea* evolution, and variability of 18S–25S loci between *Quercus* and *Cyclobalanopsis* subgenera.

Keywords Fagaceae karyotype · *Quercus* · *Castanea* · *Fagus* · rDNA · Triploid *Quercus suber*

Communicated by A. Kremer

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Introduction

The Fagaceae is an important family of forest trees in the Northern hemisphere, dominating temperate forests and Mediterranean ecosystems. It comprises ten genera (Manos et al. 2008) from which three of the most important, at the ecological and mostly at the economic level, are *Quercus* L., *Castanea* Mill, and *Fagus* L. Various species of these genera are highly exploited as a source of high-quality timber, plywood, charcoal, and cork, as well as for the production of fruits and seeds for human and animal consumption (Chalupa 1986; Mendes and Graça 2009; Singh 2010).

Quercus is the largest genus with around 400 species (Nixon 1997) subdivided in two subgenera: *Quercus*, with the majority of oaks, and *Cyclobalanopsis*, the strictly

Asian group (Camus 1936–54; Nixon 1993), although the latter was originally described as a separate genus and recently referred as an infrageneric group by Denk and Grim (2009, 2010). *Fagus* and *Castanea* are smaller genera, comprising 13 species distributed in two subgenera and seven species distributed in three sections, respectively (Denk 2003; Johnson 1988). Several authors agree on the evolutionary closeness of *Quercus* and *Castanea* (Barreneche et al. 2004; Casasoli et al. 2006; Li et al. 2004; Manos and Steele 1997; Manos et al. 2001), and on the early divergence of *Fagus* from the remaining genera of the Fagaceae (Li et al. 2004; Manos et al. 2001; Oh and Manos 2008). In the Fagaceae, several phylogenetic studies based in ribosomal intergenic regions (Denk and Grim 2010; Lang et al. 2006, 2007; Manos and Steele 1997; Manos et al. 2001) have attempted to understand the evolutionary relationships within and between genera.

Ribosomal RNAs are the markers of choice for such evolutionary studies in eukaryotes, and are encoded by two types of genes organized in tandem repeats and localized at one or more sites within the chromosome complement. The 45S rDNA sequences are found mainly clustered on the nucleolus-organizing regions (NORs), and consist of the 18S, 5.8S, and 25S rRNA genes, external transcribed spacers (ETS), internal transcribed spacers (ITS), and an intergenic spacer (IGS). The 5S rDNA repeat unit also comprises a nontranscribed spacer (NTS) which separates adjacent genes in the array. The intergenic sequences are exceptionally homogenous despite their enormous number of copies, clearly indicating that these repeats undergo a degree of concerted evolution. This mechanism has been proposed for the homogenization of rDNA sequences clusters within a species, but also allowing for sequence divergence across species (reviewed in Charlesworth et al. 1994). Incomplete concerted evolution, however, as verified in *Platanus* spp., can lead to intraindividual ITS variability (Grimm and Denk 2008).

Within species the rDNA locus number is usually stable and thus provides useful markers for chromosome identification (e.g., Doudrick et al. 1995). Nucleolus organizer and 5S rDNA loci positions however are highly polymorphic between different taxa and are well known for their potential intragenomic mobility, and are therefore considered a major force operating during speciation (Schubert 2007). The number and location of NORs can be used as chromosomal landmarks that provide valuable evidence for investigating genome evolution at both the chromosomal and molecular levels.

Divergence and speciation are often accompanied by rearrangements in chromosome complements, and thus the comparative analysis of karyotypes is an important tool for evolutionary studies (Schubert 2007). Karyotype evolution in *Arabidopsis thaliana*, for example, has resulted from large chromosome reorganizations at repeated regions, such as the terminal NORs. These phenomena led to a decrease in the number of NOR loci, accompanied by a reduction in the basic chromosome number (Lysak et al. 2006). Reduction of NOR loci has also occurred in several species during polyploidization, indicating that the loss of rDNA copies may be a common feature during genome evolution and speciation (Malinska et al. 2010; Pontes et al. 2004). Moreover, a process involving both loss and gain of rDNA loci has been proposed to have occurred during the evolution of some *Coffea* species (Hamon et al. 2009).

While intergenic spacers are rather variable between species, the rDNA genes are highly conserved between eukaryotes, even in distantly related species. Their presence is easily detected using fluorescence in situ hybridization (FISH), enabling molecular cytogenetics to be used as a tool in genome organization and evolutionary studies, as in genera such as *Allium* (Ricroch et al. 1992), *Hordeum* (Leitch and Heslop-Harrison 1993), *Aegilops* (Badaeva et al. 1996; Castilho and Heslop-Harrison 1995), *Arachis* (Raina and Mukai 1999), *Brassica* (Maluszynska and Heslop-Harrison 1993; Snowdon 2007), *Citrus* (de Moraes et al. 2007), *Coffea* (Hamon et al. 2009), and *Arabidopsis* (Pontes et al. 2004). In *Pinus*, the combination of both molecular and cytogenetic studies has shown that the variation in rDNA loci distribution has phylogenetic implications, since the similarity of FISH patterns was correlated with closeness of taxa, as reported by ITS sequence-based phylogenies (Cai et al. 2006; Hizume et al. 2002; Liu et al. 2003). Distinct rDNA FISH patterns with variable loci number and position have been observed in different *Pinus* species, although they have highly similar karyotypes (Cai et al. 2006). In contrast, several European and American species of *Quercus* subgenus *Quercus* showed extensive karyotype conservation of chromosome number, heterochromatin distribution, and rDNA FISH patterns (Zoldos et al. 1999). Three species of *Quercus* subgenus *Cyclobalanopsis* also revealed the same number of rDNA loci, although in different locations (Chokchaichamnankit et al. 2008). The karyotype knowledge of species from genera *Fagus* and *Castanea* however is very limited (Jaynes 1962; Ohri and Ahuja 1991), and additional information about rDNA mapping and genome organization is needed for these genera.

In the present work, we have studied genome organization in certain members belonging to different genera of the Fagaceae. We have mapped the rDNA loci in nine European and Asian species belonging to the genera *Fagus*, *Quercus*, and *Castanea*, for the first time, by characterizing the karyotypes of three representative species from each genus. In undertaking this work, we have addressed the following questions: (1) Does the *Quercus* genome have a conserved number of rDNA loci across its distribution

range? (2) How are the rDNA loci mapped in European and Asian *Castanea*? (3) How different is the *Fagus* genome from the other Fagaceae? And finally, (4) what do the rDNA FISH patterns tell us about evolution in the Fagaceae?

Materials and methods

Plant material

The plant material used in this study included seeds collected from individuals in natural populations, from botanical gardens or kindly provided by nurseries (Table 1). Root tips were collected from germinated acorns, or from seedlings grown in a greenhouse (22±2°C and photoperiod of 16 h).

Flow cytometry using leaves from germinated acorns was performed to confirm the occurrence of different ploidy levels, as determined by chromosome counting, in individuals of *Quercus suber* L. Leaves for the internal reference standard for genome size estimations [*Glycine max* (L.) Merr. "Polanka"] were obtained from seeds (kindly provided by the Laboratory of Molecular Cytogenetics and Cytometry, Olomouc, Czech Republic) germinated in pots in a greenhouse (22±2 C and photoperiod of 16 h).

Chromosome preparations

C-metaphases were induced by treating roots with a saturated solution of α-bromonaphthalene for 3–4 h at room temperature. Root tips were fixed in fresh ethanol to glacial acetic acid (3:1, *v*/*v*), and well-spread chromosomes were obtained using the aliquot dropping technique adapted from Zoldos et al. (1999). Briefly, the fixed root tips were first washed for 20 min in an enzyme buffer [0.03% EDTA in 2× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate in distilled water, pH adjusted to 7.0), pH adjusted to 4.2] to remove the fixative. Non-meristematic tissue was removed with a razor blade under a stereo microscope, and only the terminal portion (ca. 3 mm) without the root cap was transferred into an enzymatic mixture consisting of 2% (*w*/*v*) cellulase "Onozuka" R10 (from *Trichoderma viride*, Yakult Honsha, Japan), 3% (*v*/*v*) pectinase (from *Aspergillus niger*, Sigma, St. Louis, MO, USA), 0.3% pectolyase Y-23 (from *Aspergillus japonicus*, Sigma), and 0.03% EDTA in 2× SSC buffer with pH adjusted to 4.2. The suspension was incubated at 37°C for 3 h for partial digestion of the cell walls. The material was completely dispersed with a micropipette and further incubated for additional 30 min at 37°C. The cell suspension of one seminal root tip, or of three adventitious root tips, was then centrifuged at 800×*g* for 5 min. The supernatant was then removed, and the cellular mass was washed twice in enzyme buffer, then twice in fresh ice-cold ethanol to glacial acetic acid (3:1, *v*/*v*) fixative, with decreasing centrifugations (from 800×*g* to 400×*g*, 5 min each). Finally, the pellet was resuspended in an appropriate volume of fixative. Approximately 20 μl of chromosome suspension was dropped onto 3-aminopropyltriethoxysilane-coated slides. Slides were then air-dried and screened with phase contrast microscopy to check cell density and c-metaphase quality. When cytoplasm was abundant, the fixed suspension was further centrifuged, and the fixative replaced by fresh 60% glacial acetic acid during 1 min for cytoplasm removal. After centrifugation the pellet was resuspended once more in an appropriate volume of fixative, followed by the aliquot dropping procedure.

DNA:DNA fluorescent in situ hybridization

The pTa71 clone, a 9-kb fragment from wheat containing part of the 18S and the entire 5.8S and 25S coding regions, together with nontranscribed spacers (Gerlach and Bedbrook 1979), was used as an 18S–25S rDNA probe labelled with digoxigenin, or biotin, by nick translation. The clone pTa794, which contains a complete 410-bp *Bam*HI fragment of the 5S rRNA gene and spacer regions from wheat (Gerlach and

Table 1 Fagaceae species studied and the respective source of plant material

Species	Source of plant material
F. sylvatica L.	St. Isidro nursery, Portugal
Q. suber L.	Lisbon, Portugal
Q. ilex subsp. *rotundifolia* (Lam.) O.Schwarz ex Tab. Morais	Lisbon, Portugal
Q. sessilifolia Blume	TFRI, Taipei, Taiwan
Q. glauca Thunb.	TFRI, Taipei, Taiwan
Q. acutissima Carruth.	Tsukuba, Japan
Q. serrata Murray	Tsukuba, Japan
C. crenata Siebold and Zucc.	Tsukuba, Japan
C. sativa Mill.	Trás-os-Montes, Portugal
C. mollissima Blume	UTAD Botanical Garden, Portugal

Dyer 1980), was used as a 5S rDNA probe and labelled by polymerase chain reaction (PCR) with digoxigenin or biotin. Both probes were used in all species except in *Q. suber*. In this species a specific probe (Qsu5S) was produced by PCR amplification of a 353-bp fragment (including 89 bp of the conserved gene sequence and 264 bp of the NTS) of the repeated 5S rDNA sequence from genomic DNA of *Q. suber* with the following primers: 5′-ATCCCATCAGAACTCCG-3′/5′-GCAACGATGCTCCTTAA-3′. Probes labelled with digoxigenin were detected with anti-digoxigenin antibody conjugated to fluorescein isothiocyanate, and biotin-labelled probes were detected with streptavidin conjugated to Cy3. DNA was counterstained with DAPI in CITIFLUOR antifade buffer (AF1; Agar Scientific, UK).

Cell analysis and image acquisition

Hybridized slides were analysed with an epifluorescence microscope (Axioskop 2; Zeiss, Jena, Germany). All images were collected using an AxioCam digital camera (Zeiss) controlled by AxioVision 3.0, and were assembled using Adobe Photoshop 6.0. Measurements were performed in individualized straight metaphase DAPI-stained chromosomes from more than five individuals for each species (*Fagus sylvatica* L., *Q. suber*, and *Castanea sativa* Mill.), using the AxioVision measurement module 3.0.0 (Zeiss).

Data were then exported to an Excel (Microsoft® Office Excel 2003, USA) spreadsheet, and homologues pairs of each complement were identified through their total length and centromeric index (CI=length of the short arm/total length×100), as well as by mapping rDNA FISH landmarks when available. Mean and standard deviations were calculated for all metaphase pairs of each species. Nomenclature of the chromosomes followed that of Levan et al. (1964): m (centromere at median point), m (centromere at median region), sm (centromere at submedian region), and st (centromere at subterminal region).

Genome size estimation using flow cytometry

Nuclear suspensions were prepared according to Galbraith et al. (1983) using the woody plant buffer (WPB, Loureiro et al. 2007). Briefly, 100 mg of *Q. suber* leaves and 50 mg of leaves from the reference standard (*G. max*) were co-chopped with a sharp razor blade in 1 ml of WPB [0.5 mM spermine·4HCl, 30 mM sodium citrate·$3H_2O$, 20 mM MOPS, 80 mM KCl, 20 mM NaCl, and 0.5% (*v*/*v*) Triton X-100, pH adjusted to 7.0]. Due to the high levels of phenolic compounds present in *Q. suber* leaves, it was essential to significantly reduce the chopping intensity. The nuclear suspension was recovered and filtered through a 50-mm nylon filter to remove cell fragments and large debris. Nuclei were stained with 50 mg ml^{-1} propidium iodide (PI; Fluka, Buchs, Switzerland), and 50 mg ml^{-1} RNase (Sigma) was added to the nuclear suspension to prevent staining of double-stranded RNA. Samples were incubated on ice and analysed within 5 min in the SYSTEM II software (v. 3.0, Coulter Electronics, Hialeah, FL, USA). Nuclear samples were analysed using a Beckman Coulter Epics-XL (Coulter Electronics) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. PI fluorescence was collected through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. The amplification system was set to a constant voltage and gain throughout the experiment. Three replicates per individual were done on three different days (one replicate per day), to reduce the possible effect of instrumental drift on the estimations. Only histograms with G_0/G_1 peaks below 4.0% (mean CV value of 2.4% for the G_0/G_1 peak of *Q. suber*) were considered a valid replicate.

Results

Karyotypes of *F. sylvatica*, *Q. suber*, and *C. sativa* were established using the chromosome classification system of Levan et al. (1964) and were based on centromeric index values along with rDNA loci mapping using FISH (Tables 2, 3, and 4), and the number of rDNA loci and their locations were determined in another seven European and Asian species belonging to *Quercus* and *Castanea* genera.

All the studied individuals of each species, except *Q. suber*, presented the same chromosome number, i.e., 2n=2*x*=24. In *Q. suber*, one individual was triploid (2n=3*x*=36 chromosomes), while all the others were found to be diploid. Considering only diploid individuals, four distinct patterns of rDNA mapping were detected (Fig. 1). One pattern is observed in *F. sylvatica* which presents two 5S rDNA pericentromeric loci and four terminal 18S–25S rDNA loci. The 5S loci are located at the short arm of the two largest chromosomes with the centromere at the median region (Table 2), while 18S–25S rDNA loci are present on the short arms of chromosomes 3, 5, 6, and 11, which have, with the exception of the fifth pair, the centromere at the submedian region (Table 2, Figs. 1a and 2). The Asian *Quercus sessilifolia* Blume, belonging to the subgenus *Cyclobalanopsis*, gave a different rDNA FISH pattern, with only one 5S rDNA pericentromeric locus in the short arm of one of the largest chromosomes and five 18S–25S rDNA loci, two pericentromeric, and three in terminal positions (Figs. 1b and 2). In a different way, *Quercus glauca* Thunb., which also belongs to this subgenus, has a third distinct rDNA loci mapping pattern: it is identical to the one observed in *Quercus* subgenus *Quercus* and in *C. sativa* and *Castanea crenata* Siebold and Zucc. All these species show one pericentromeric 5S rDNA locus on the short arm of one

Table 2 Morphometric data from 5 c-metaphase cells of *F. sylvatica*

Chromosome pair[a]	Number (counts)[b]	Total length (μm)		Short-arm length (μm)	Centromeric index, %[c]	Chromosome type	FISH signal	
		Range	Mean±SD	Mean±SD	Mean±SD		18S–25S	5S
1	9	1.88–2.18	2.02±0.15	0.94±0.09	46.46±1.99	m		S
2	9	1.72–2.04	1.92±0.14	0.85±0.08	44.15±1.44	m		S
3	8	1.67–1.92	1.81±0.11	0.65±0.04	35.86±0.75	sm	S	
4	8	1.65–1.90	1.80±0.13	0.74±0.07	41.16±0.97	m		
5	8	1.60–1.88	1.75±0.13	0.74±0.08	42.01±1.47	m	S	
6	9	1.58–1.80	1.71±0.12	0.63±0.05	36.41±1.32	sm	S	
7	10	1.48–1.78	1.63±0.11	0.80±0.07	49.77±0.19	M		
8	9	1.46–1.70	1.59±0.10	0.64±0.05	40.29±0.74	m		
9	10	1.37–1.64	1.47±0.09	0.55±0.04	37.53±0.62	sm		
10	9	1.13–1.59	1.32±0.22	0.49±0.09	36.83±0.76	sm		
11	9	1.12–1.44	1.28±0.13	0.37±0.06	28.80±2.06	sm	S	
12	10	1.07–1.30	1.20±0.09	0.53±0.05	44.13±1.12	m		

SD standard deviation, *S* short arm of the chromosome, *M* centromeric position is sensu stricto median, *m* centromeric position is in the median region, *sm* centromeric position is in the submedian region

[a] Chromosomes were ordered and numbered according to their rank of the total length at metaphase (full contraction)

[b] Only chromosomes totally visible and not too folded were taken into account

[c] Centromeric index=100×(length of the short arm/total length)

of the largest median chromosomes and two 18S–25S rDNA loci (one major and terminal and one minor and pericentromeric) on short arms of distinct chromosomes (Figs. 1d and 2). A detailed comparison of *Q. suber* and *C. sativa* karyotypes (Tables 3 and 4) reveals, moreover, that in *Q. suber*, as well as in other *Quercus* species of the same subgenus, the 5S rDNA locus is located on chromosome 2 of the second largest chromosome pair, with the centromere at the median region (Table 3); whereas in *C. sativa* and *C. crenata*, this locus is positioned on chromosome 3 (Table 4, Fig. 2). Differences on 18S–25S rDNA loci chromosome mapping were also detected between *Q. suber* and *C.*

Table 3 Morphometric data from 10 c-metaphase cells of *Q. suber*

Chromosome pair[a]	Number (counts)[b]	Total length (μm)		Short-arm length (μm)	Centromeric index, %[c]	Chromosome type	FISH signal	
		Range	Mean±SD	Mean±SD	Mean±SD		18S–25S	5S
1	19	2.55–3.49	2.94±0.34	1.38±0.19	46.86±2.20	m		
2	20	2.30–2.81	2.55±0.15	1.10±0.07	43.14±1.94	m		S
3	19	1.97–2.67	2.31±0.22	0.85±0.08	36.74±0.81	sm		
4	18	1.88–2.61	2.30±0.28	0.77±0.15	33.16±3.98	sm	S	
5	16	1.80–2.53	2.15±0.22	0.90±0.08	42.01±1.47	m		
6	19	1.72–2.30	2.00±0.12	0.73±0.05	36.41±1.32	sm		
7	19	1.62–2.28	1.93±0.17	0.85±0.09	43.74±1.49	m	S	
8	16	1.62–2.21	1.85±0.20	0.90±0.11	49.30±0.51	M		
9	20	1.50–1.99	1.77±0.13	0.64±0.05	35.96±1.65	sm		
10	18	1.40–1.97	1.69±0.18	0.82±0.08	49.46±1.31	M		
11	19	1.30–1.89	1.59±0.20	0.56±0.05	35.59±1.94	sm		
12	17	1.20–1.83	1.52±0.21	0.72±0.12	46.68±1.89	m		

SD standard deviation, *S* short arm of the chromosome, *M* centromeric position is sensu stricto median, *m* centromeric position is in the median region, *sm* centromeric position is in the submedian region

[a] Chromosomes were ordered and numbered according to their rank of the total length at metaphase (full contraction)

[b] Only chromosomes totally visible and not too folded were taken into account

[c] Centromeric index=100×(length of the short arm/total length)

Table 4 Morphometric data from 9 c-metaphase cells of *C. sativa*

Chromosome pair[a]	Number (counts)[b]	Total length (μm)		Short-arm length (μm)	Centromeric index, %[c]	Chromosome type	FISH signal	
		Range	Mean±SD	Mean±SD	Mean±SD		18S–25S	5S
1	16	1.84–3.73	2.98±0.61	1.44±0.31	47.78±1.60	m		
2	15	1.58–3.74	2.94±0.64	1.34±0.34	45.61±2.83	m	S	
3	14	1.55–3.34	2.62±0.56	1.13±0.26	42.98±2.03	m		S
4	15	1.47–3.07	2.41±0.47	1.07±0.20	44.20±2.10	m	S	
5	15	1.35–2.83	2.12±0.51	0.83±0.20	39.04±0.37	m		
6	15	1.16–2.61	1.99±0.49	0.86±0.21	43.88±1.77	m		
7	12	1.28–2.33	1.92±0.41	0.95±0.21	49.46±0.59	M		
8	16	1.18–2.33	1.90±0.44	0.81±0.20	42.53±1.05	m		
9	15	1.13–2.33	1.82±0.47	0.68±0.17	36.84±1.47	sm		
10	16	1.11–2.22	1.74±0.39	0.80±0.17	46.01±2.28	m		
11	15	1.12–2.21	1.70±0.34	0.60±0.14	34.96±2.74	sm		
12	16	1.07–2.13	1.68±0.31	0.78±0.14	34.96±2.74	m		

SD standard deviation, *S* short arm of the chromosome, *M* centromeric position is sensu stricto median, *m* centromeric position is in the median region, *sm* centromeric position is in the submedian region

[a] Chromosomes were ordered and numbered according to their rank of the total length at metaphase (full contraction)

[b] Only chromosomes totally visible and not too folded were taken into account

[c] Centromeric index = 100 × (length of the short arm/total length)

sativa: in *Q. suber*, the terminal 18S–25S rDNA locus is located on the submedian chromosome 4, while in *C. sativa* it is present on the median chromosome 2; and the pericentromeric 18S–25S rDNA locus is located on chromosome 7 in *Q. suber* (Table 3) and on chromosome 4 in *C. sativa* (Table 4), both with centromeres at the median region (Tables 3 and 4). Conversely, *Castanea*

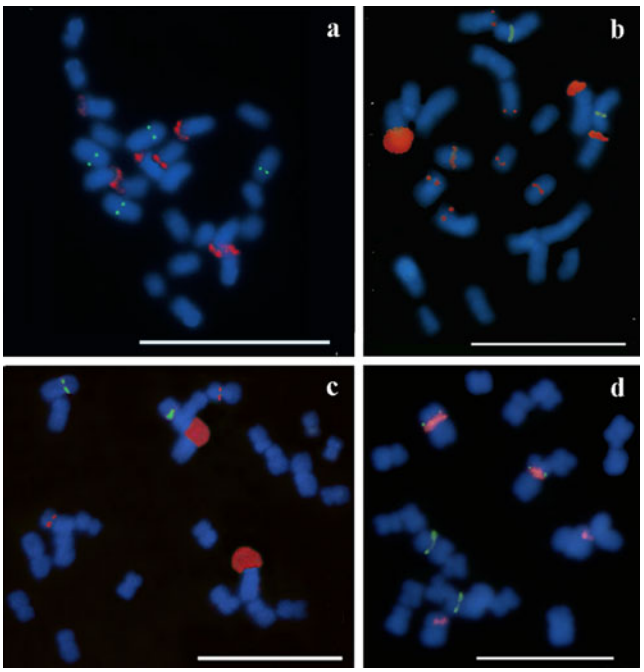

Fig. 1 Fluorescent in situ hybridization showing four rDNA physical mapping patterns. Complete c-metaphases of *F. sylvatica* (**a**), *Q. sessilifolia* (**b**), *C. sativa* (**c**), and *C. mollissima* (**d**) with 24 chromosomes. The 5S and 18S–25S rDNA loci are mapped in *green* and *red*, respectively. Chromosomes are counterstained by DAPI. *Bars* 10 μm

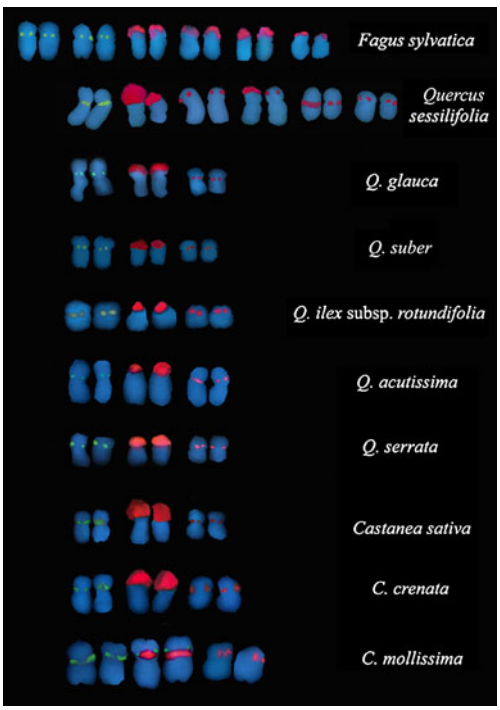

Fig. 2 Homologous chromosomes pairs bearing rDNA loci of European and Asian Fagaceae species. 5S rDNA loci are presented in *green* and the 18S–25S rDNA loci are in *red*

mollissima Blume reveals a fourth pattern, showing two 5S and two 18S–25S rDNA loci on three distinct chromosomes: one chromosome with a 5S rDNA pericentromeric locus, another one with a 18S–25S rDNA pericentromeric locus and a third one with a centromere proximal 18S–25S rDNA and a distal 5S locus (Figs. 1d and 2).

In species presenting only two 18S–25S rDNA loci, the terminal ones are always the major NORs, revealing a huge amount of rDNA sequence with a striking polymorphism between both loci (Fig. 2), and sometimes accentuated major NORs heteromorphism, as exemplified by *Q. sessilifolia* and *Quercus acutissima* Carruth. (Fig. 2). On the other hand, all the *F. sylvatica* 18S–25S rDNA loci display approximately the same signal intensity, with no marked differences between them. Conversely, in all species, the 5S rDNA loci present a constant pericentromeric location with similar signals intensities between species.

As mentioned above, one triploid individual of *Q. suber* with 36 chromosomes was found, with a whole additional set of chromosomes and the respective additional number of rDNA loci (Fig. 3). The triploid nature of this plant was further confirmed by measuring its nuclear DNA content by flow cytometry, and comparing it with the nuclear DNA content of diploid individuals. While diploid individuals of *Q. suber* had a mean 2C value of 1.87±0.039 pg (n=7, R=3), the polyploid gave a 2C value of 2.75±0.01 pg (n=1, R=2), which corresponds to 1.48 times more DNA, confirming its triploid nature (Table 5, Fig. 4).

Discussion

The Fagaceae family comprises thousands of species belonging to ten genera (Manos et al. 2008). All the species share the same chromosome number (2n=2x=24), except *Trigonobalanus doichangensis* (A. Camus) Forman (2n=2x=14, Chen et al. 2007) and *Trigonobalanus verticillata* (2n=2x=42, Hou 1971). In order to understand genome organization and karyotype variation in this family, species belonging to three different genera covering distinct geographic locations, from Europe to Asia, were studied using rDNA physical mapping and chromosome morphometric analysis. Indeed, we reveal for the first time the physical mapping of 18S–25S and 5S rDNA loci for nine species from three different genera.

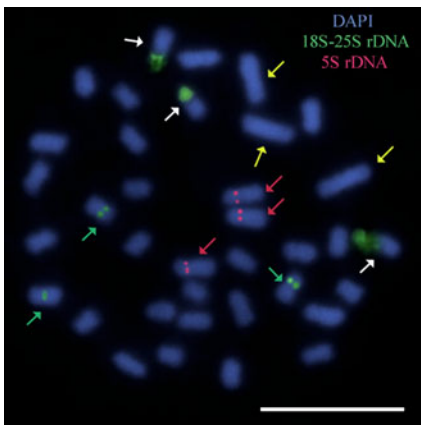

Fig. 3 Fluorescent in situ hybridization in root c-metaphase of a triploid individual of *Q. suber* 2n=3x=36, using ribosomal DNA probes (pTa71 and 5SQsu) as FISH markers for detection of homologous chromosomes. The four most easily detectable triplets of homologous chromosomes are evidenced by the *arrows of the same color*. *Bar* 10 μm

Table 5 Estimation of absolute DNA nuclear content in *Q. suber* individuals

Ploidy level	Nuclear DNA content			
	2C±SD (pg)	2C (Mbp)[a]	CV (%)	n (R)
Q. suber				
2n=2x=24	1.87±0.039	1,826	3.06	7 (3)
Q. suber				
2n=3x=36	2.75±0.010	2,690	2.84	1 (2)

The values are given as mean and standard deviation of the nuclear DNA content (2C in picograms and megabase pairs) of the individuals of each ploidy level of *Q. suber*. The mean coefficient of variation of G_0/G_1 peaks (CV, in percent) and the number of analysed individuals (n) and replicates (R) are also provided for each ploidy level detected in *Q. suber*. The replicates were made on three different days

[a] 1 pg DNA=978 Mbp (Doležel et al. 2003)

The karyotypes enabled us to recognize three homologous chromosome pairs in *Q. suber* and *C. sativa*, supporting the idea of karyotype conservation between these species, as proposed by Casasoli et al. (2006) for *Quercus robur* and *C. sativa*. With respect to rDNA physical mapping, although *Fagus*, *Quercus*, and *Castanea* show the same chromosome number (2n=2x=24), these genera exhibit variations in the number and location of 18S–25S and 5S loci rDNA, as well as in the number of rDNA repeats per locus. Such variability is new in Fagaceae, as the studies developed so far in other *Quercus* species, and in genera *Castanopsis* and *Lithocarpus* from different geographic locations (Chokchaichamnankit et al. 2008; Zoldos et al. 1999), always showed two 18S–25S rDNA loci and one 5S rDNA locus, although with diverse chromosome locations. All of the analysed species show terminal NORs, except *C. mollissima* that has only intercalary 18S–25S rDNA, which to our knowledge constitutes a novelty in the Fagaceae. Furthermore, *F. sylvatica* and *Q. sessilifolia* have the highest number of these loci ever detected in the family (four and five, respectively). In contrast, 5S rDNA loci maintain a pericentromeric location in all the species studied so far (our results and those from Chokchaichamnankit et al. 2008 and Zoldos et al. 1999).

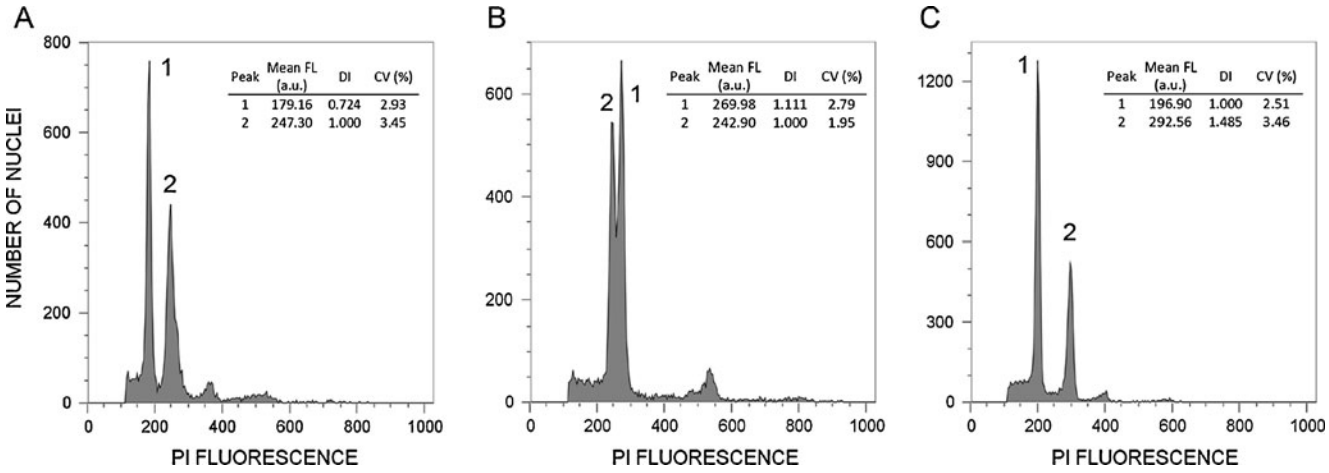

Fig. 4 Flow cytometric histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from the internal reference standard (*peak 2*; *G. max* with 2C=2.5 pg DNA) and the *Q. suber* L. (*peak 1*). **a** Diploid individual (2n=2*x*=24) and **b** triploid individual (2n=3*x*=36). **c** Histogram obtained after simultaneous analysis of nuclei isolated from a diploid individual (*peak 1*) and the triploid individual (*peak 2*). The mean channel number (*mean FL*, a.u., arbitrary units), DNA index (*DI*=mean channel number of sample/mean channel number of reference standard), and coefficient of variation value (*CV*, in percent) of each peak are also given

A detailed overview of the results obtained in each genus studied reveals that *Castanea* presents a certain degree of variability within its rDNA loci, while European and Japanese chestnuts share the same rDNA number and locations. Chinese species show a drastic change in the position of the major NOR, from terminal (as in the other Fagaceae species) to interstitial; and an extra 5S locus was also detected near the NOR. The presence of an additional 5S rDNA locus has also been reported in the genus *Coffea* (Hamon et al. 2009), while variations in the number and locations of these genes have been described in several plant families, such in Triticeae where the presence and order of the 5S and 18S–25S rDNA sequences on groups 1 and 5 can vary between species (Castilho and Heslop-Harrison 1995). All these findings suggest the capacity of these sequences to move across the genome, hypothetically as a result of chromosome rearrangements, unequal crossing over, and transposition events (Hamon et al. 2009; Sheng and Wang 2010).

With respect to the genus *Quercus*, we mapped the rDNA sequences in four European and Asian species belonging to subgenus *Quercus*, and found the same rDNA number and location in all the species studied. This pattern was also found in other European and North American *Quercus* species (Zoldos et al. 1999), indicating a possible stabilization of their karyotypes despite the high level of radiation present in this genus, and even considering the refuge geographic area of the Iberian Peninsula. This high conservation of karyoptypes could account for the vast number of fertile hybrids occurring naturally between *Quercus* species (Petit et al. 2004), which in turn has been proposed to contribute to the evolutionary processes in oaks, as in *Quercus afares* (Mir et al. 2006). Besides interspecific hybridization, polyploidization has also been detected previously in some *Quercus* species. Here we report for the first time a true *Q. suber* triploid, the presence of which points to a previous autopolyploidization event, similar to that observed in other *Quercus* species (Burda and Shchepotiev 1973; Butorina 1993, Dzialuk et al. 2007). Moreover, the nuclear DNA content of *Q. suber* diploids was similar to other C values available in the literature (Loureiro et al. 2005; Zoldos et al. 1998).

In contrast to the subgenus *Quercus*, other rDNA FISH patterns observed point to the occurrence of variability in the subgenus *Cyclobalanopsis*, whereas, besides the pattern already described for the first subgenus *Q. glauca*, up to five 18S–25S rDNA loci were found in *Q. sessilifolia*. The position of the subgenus *Cyclobalanopsis* within *Quercus* has been a subject of several interpretations during the last decade (Manos et al. 2001, 2008; Oh and Manos 2008). We hypothesize that some of these divergences can be correlated with the different number of rDNA loci present in this group, where different FISH patterns are found: either the same, as in all species of subgenus *Quercus* and other *Cyclobalanopsis* species (Chokchaichamnankit et al. 2008), or different with a high number of paralogous sites. Moreover, Denk and Grimm (2010), based on ITS and 5S-IGS data, concluded that the *Cyclobalanopsis* group shares a common ancestor with the *Cerris* and *Ilex* groups, and at the same time is closer to other Fagaceae than to the remainder of species of the *Quercus* genus. Interestingly, these phylogenetic patterns indicate that the *Cyclobalanopsis* group was isolated, and possibly radiated soon after the initial divergence, separating the two major clades within oaks. This scenario, taken altogether with our data, suggests that genome evolution in the genus *Quercus* was accompanied by changes in the number of the 18S–25S rDNA loci.

F. sylvatica represents the third genus studied and is considered the basal genus of the family. This species has two intercalary 5S and four terminal 18S–25S rDNA loci, which constitutes a unique pattern among the studied species. *Fagus* is known for displaying high intraspecific and intraindividual ITS polymorphism (Grimm et al. 2007), probably originated from paralogous variants, which is in accordance with the several number of terminal rDNA loci revealed by FISH for *F. sylvatica*. Incomplete concerted evolution at the genomic level has been proposed to occur in this genus (Denk et al. 2005), which is at odds with the proposal of Zhang and Sang (1999) that the terminal location of rDNA loci may have facilitated the process of sequence homogenization in peonies through unequal crossing over.

Considering the difficulty of distinguishing orthologs from paralogs among rDNA loci, using molecular analyses, and subsequent risks in relying exclusively on rDNA sequences for phylogenetic inference, FISH has undoubted importance for phylogenetic studies (Alvarez and Wendel 2003). Indeed, an understanding of the number and location of rDNA sequences may facilitate the evaluation of alternative explanations for "missing sequences," such as locus loss and homogenization (Li and Zhang 2002). In conclusion, our results suggest that divergence and speciation in the Fagaceae was accompanied by genome restructuring, and that reductions and possible gains in rDNA loci provided one of the mechanisms.

Acknowledgments The authors would like to thank Dr. Hachemi Merouani, Dr. Atshuchi Sakai, Prof. Carlos Abreu, Engr. Isabel Silvestre, Engr. Carla Faria, and Bruno Larsen for generously providing help in obtaining and preserving the plant material. We thank Prof. Wanda Viegas for many helpful comments. We are also very grateful to Prof. Neil Jones for his critical revision of the manuscript and editing of English. T.R was supported by Fundação Ciência e Tecnologia, Portugal (Grant SFRH/BD/13319/2003).

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