Dancing together and separate again: gymnosperms exhibit frequent changes of fundamental 5S and 35S rRNA gene (rDNA) organisation

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In higher eukaryotes, the 5S rRNA genes occur in tandem units and are arranged either separately (S-type arrangement) or linked to other repeated genes, in most cases to rDNA locus encoding 18S–5.8S–26S genes (L-type arrangement). Here we used Southern blot hybridisation, PCR and sequencing approaches to analyse genomic organisation of rRNA genes in all large gymnosperm groups, including Coniferales, Ginkgoales, Gnetales and Cycadales. The data are provided for 27 species (21 genera). The 5S units linked to the 35S rDNA units occur in some but not all Gnetales, Coniferales and in Ginkgo (30% of the species analysed), while the remaining exhibit separate organisation. The linked 5S rRNA genes may occur as single-copy insertions or as short tandems embedded in the 26S–18S rDNA intergenic spacer (IGS). The 5S transcript may be encoded by the same (Ginkgo, Ephedra) or opposite (Podocarpus) DNA strand as the 18S–5.8S–26S genes. In addition, pseudogenised 5S copies were also found in some IGS types. Both L- and S-type units have been largely homogenised across the genomes. Phylogenetic relationships based on the comparison of 5S coding sequences suggest that the 5S genes independently inserted IGS at least three times in the course of gymnosperm evolution. Frequent transpositions and rearrangements of basic units indicate relaxed selection pressures imposed on genomic organisation of 5S genes in plants. 

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INTRODUCTION

Organisation of ribosomal DNA in plants

The four types of ribosomal RNA (18S, 5.8S, 26S and 5S) are essential constituents of the ribosomes of all euukaryotes, encoded by variable numbers of copies of rRNA genes, called rDNA. Plants are known to bear an extraordinarily high number of these genes (from 1000 to more than 30,000) arranged in long tandem arrays on chromosomes (Hemleben and Zentgraf, 1994). The 18S–5.8S–26S genes are usually organised, in that order, in a single operon, in one or several chromosomal loci. In cell nuclei, the genes are cotranscribed by RNA polymerase I into a primary transcript, which is shorter in plants (35S rRNA) than in animals (45S rRNA) (Seitz and Seitz, 1979). We will therefore refer to 35S rDNA throughout the work. The 5S is the only rRNA gene that is transcribed separately, by RNA polymerase III in nucleoplasm. The 5S rDNA typically occurs at positions separate from 35S rDNA in multicellular euukaryotic organisms, sometimes forming long arrays of tandemly arranged genes spaced by intergenic spacers (IGS) of variable lengths. Nevertheless, there are several exceptions to this rule, for example, 5S gene linkage to other repetitive gene families including 35S, small nuclear RNAs, histone genes or the trans-spliced leader (Drouin and de Sa, 1995), which were formerly believed to represent transition states between linked (typically prokaryotic) and unlinked (typically eukaryotic) arrangement. Consistent with this assumption, 5S rRNA genes in plants were thought to be mostly arranged separately from 35S rDNA (S-type arrangement), organised in tandem repeats whose number varied from less than 1000 to over 75,000 (Campbell et al., 1992; Sastri et al., 1992).

Regardless, several lines of evidence indicate that the transition hypothesis may not work for green plants, at least. Earlier independent studies showed 5S gene integration into the 26S–18S spacers in the moss Funaria hygrometrica and the liverwort Marchantia polymorpha (Sone et al., 1999). In the recent genome-wide assessment of rDNA organisation in land plants done by Wicke et al. (2011), it was concluded that a linked 35S–5S rDNA (L-type arrangement) should be regarded as the ancestral state as this type has been observed in streptophyte algae and early diverging green plants such as liverworts, mosses, hornworts, lycophytes and monilophytes. However, linkage between SS and 35S genes was also found in several angiosperm genera such as the phylogenetically derived genus Artemisia (Garcia et al., 2009). Later, deeper molecular and cytogenetic studies revealed that the L-type arrangement could be present in 25% of the Asteraceae family (Garcia et al., 2010; Mazzella et al., 2010).

Gymnosperms: a rather unexplored field for rDNA research

Although showing a wide diversity in morphology and ecology, gymnosperms consist of only 14 families with roughly 1000 species, a derisory number when compared with the estimated number of angiosperms or even with some of its largest families (such as Asteraceae and Orchidaceae, believed to hold more than 20,000 species each). There are four classically considered gymnosperm
orders (Figure 1): (i) Coniferales are the largest, with around 650 species (division Pinophyta, including Pinaceae and the non-Pinaceae Pinophyta, frequently considered as a division on its own and named cupressophytes); (ii) Cycadales, with three extant families and about 130 species; (iii) Gnetales with three monogenic families, including 70 species; and lastly (iv) Ginkgoales, with a single extant species, *Ginkgo biloba*. Phylogenetic relationships between gymnosperm families remain questionable (see Supplementary Figure S1 for a summary on available hypotheses on gymnosperm phylogeny), to the extent that even its monophyly and if a common ancestor is shared with flowering plants is still debated. Ecological, genetic and epigenetic factors might have acted distinctly to shape angiosperm and gymnosperm genomes (Leitch and Leitch, 2012).

Cytogenetic studies carried out mostly in Pinaceae (Pinophyta) revealed separate arrangement of SS and 35S genes (Table 1). The situation outside this family is less clear. Cytogenetic studies using rDNA fluorescence in situ hybridisation revealed colocalisation of 35S and SS signals in Podocarpus (Podocarpaceae, Murray et al., 2002) and some Cupressaceae (Hizume et al., 1999), both non-Pinaceae conifers from the division Pinophyta and also in *G. biloba* (Nakao et al., 2005). In most cases, however, because of low resolution of cytogenetic methods, it is not certain whether the 35S and SS genes are physically linked or whether they form distinct arrays close to each other. There have been several molecular studies of SS rDNA in gymnosperms with the aim of determining rDNA organisation at the unit level. Out of these, independent 5S clusters were confirmed in *Pinus* (Mashkova et al., 1990; Gorman et al., 1992; Moran et al., 1992; Liu et al., 2003a), *Abies* (Besendorfer et al., 2005), *Larix* (Trentin et al., 1999), *Picea* (Brown and Carlson, 1997) and *Pseudotsuga* (Amarasinghe and Hollick, 1998). PCR screens and sequence analysis revealed S-type rDNAs in *Cycas, Ginkgo* and *Gnetum* (Wicke et al., 2011). On the basis of these numerous studies, it has been proposed that of resolution of cytogenetic methods, the 5S repeats also hold the separated configuration typical for vascular plants (Wicke et al., 2011). Conversely, Galiañ et al. (2012) undertaking a PCR-based strategy promptly isolated clones from *G. biloba* with 5S genes integrated in the 26S–18S spacer. The potentially functional 5S gene was located ~2 kb downstream of the 26S gene. However, there were also clones without 5S genes pointing to some heterogeneity of rDNA arrays. Although in situ hybridisation results favour a dominant L-type arrangement, it remains unclear as to what is the proportion of S- and L-type units in the *Ginkgo* genome.

Taking into account the relative paucity of knowledge about rDNA organisation in most gymnosperms despite the rather reduced size of the group (1000 gymnosperm versus 250 000 angiosperm species), we aim to tackle the global picture on rDNA arrangement in all major gymnosperm clades by studying its organisation in selected representatives through the use of molecular methods. This will enable an update of the state-of-art of rDNA linkages across land plants and the comparison of the different kinds of 5S insertions in all the groups studied up to now.

**MATERIALS AND METHODS**

**Plant sampling**
Fresh leaves from 27 species (21 genera), representing 9 families and all major gymnosperm groups, were obtained either from trees at the Botanical Garden of Barcelona, through plants grown at the greenhouse of the Botanical Institute of Barcelona or from trees planted in public gardens (Garden ‘Palau de Pedralbes’ and Pius XII Square gardening, Barcelona). Table 2 summarises the details of the materials collection.

**DNA extraction**
We extracted the total genomic DNA using either the CTAB method of Doyle and Doyle (1987) or the Nucleospin Plant II kit (Macherey-Nagel, GmbH et Co, Düren, Germany), depending on the quality of the vegetal material, either from silica gel-dried leaves or directly from fresh leaves. The final product was dissolved in Tris EDTA buffer. Optical density readings of the DNA concentration were estimated at an optical density of 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

**Southern blot hybridisation**
Purified genomic DNAs were digested with restriction enzymes (*BamHI, BglII, EcoRI* and *EcoRV* depending on the experiment) and separated by gel electrophoresis on a 0.9% (w/v) agarose gel. After electrophoresis, the gels were alkali blotted onto Hybond-XL membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and hybridised with 32P-labelled DNA probes (DekaLabel kit, MBI, Fermentas, Vilnius, Lithuania) as described in Garcia et al. (2009). After washing under high-stringency conditions, the hybridisation bands were visualised with a PhosphorImager (Typhoon 9410, GE Healthcare, Piscataway, NJ, USA) and the data were processed by ImageQuant software (GE Healthcare). The probes were a 220-bp PCR product derived from the 3' end of the 26S rRNA gene of tobacco (Lim et al., 2000), a cloned ~120 bp of the 5S genomic region, also from tobacco (Fulneck et al., 2002) and an 18S probe from tomato (Kiss et al., 1989). The IG51-5S probe was a 380-bp fragment from Ginkgo prepared by cutting the insert of a pGIN2 plasmid construct with EcoRV.

![Figure 1](image-url) A proposed organisation of 5S–35S genes in gymnosperm phylogeny. The support for the hypothesis comes from the works listed in Table 1. Grey lines—L-type arrangement; black lines—S-type arrangement. Grey arrows show putative integration events of 5S genes into the 26S–18S IGS. Dashed lines—putative ancestors having either L- of S-type units. The phylogeny was adapted from the currently most-accepted gnepine hypothesis—see Mathews (2009). Phylogeny of cupressophytes (non-Pinaceae conifers) adopted from Chaw et al. (1997).
**Table 1** Summary linking Southern blot data obtained in this study with previous FISH results

| Order–family | Southern blot | FISH | Source |
|--------------|---------------|------|--------|
| Coniferales (Pinophyta) | | | |
| Abies | S-type | S-type | Puizina et al., 2008 |
| Cedrus | S-type | — | |
| Larix | S-type | S-type | Lubaretz et al., 1996; Liu et al., 2006; Zhang et al., 2010 |
| Picea | S-type | S-type | Lubaretz et al., 1996; Brown and Carlson, 1997; Hizume et al., 1999; Slijak-Yakovlev et al., 2002; Shibata and Hizume, 2008 |
| Pinus | S-type | S-type | Cullis et al., 1988; Gorman et al., 1992; Karvonnen and Savolainen, 1993; Doudrick et al., 1995; Lubaretz et al., 1996; Hizume et al., 2002; Liu et al., 2003b; Cai et al., 2006; Bogunic et al., 2011 |
| Pseudotsuga | — | S-type | Hizume and Akiyama, 1992; Amarasinghe and Carlson, 1998 |
| Coniferales (non-Pinaceae Pinophyta or cupressophytes) | | | |
| Araucariaceae | Araucaria | S-type | — |
| Cupressaceae | Caliixis | S-type | — |
| | Calocedrus | S-type | — |
| | Cryptomeria | — | L-type | Hizume et al., 1999 |
| | Cunninghamia | — | L-type | Hizume et al., 1999 |
| | Cupressus | S-type | — |
| | Juniperus | S-type | — |
| | Sequoia | S-type | — |
| | Tetracislin | S-type | — |
| Podocarpaceae | Afrocarpus | L-type | — |
| | Podocarpus | L-type | L-type | Murray et al., 2002 |
| Taxaceae | Taxus | S-type | — |
| Cypodales | Cercidaceae | Cypis | S-type | — |
| | Zamiaceae | Ceratozamia | — | S-type | Tagashira and Kondo, 2001 |
| | | Encephalartos | S-type | — |
| | | Macrozamia | S-type | — |
| | | Zamia | S-type | S-type | Tagashira and Kondo, 2001 |
| Ginkgoales | Ginkgo | L-type | L-type | Hizume et al., 1999; Nakao et al., 2005; Galián et al., 2012 |
| Gnetales | Ephedraceae | L-type | — |
| | Gnetaceae | Gnetum | S-type | — |

Abbreviation: FISH, fluorescence in situ hybridisation. Data compiled through the plant rDNA database (www.plantrDNAdatabase.com; Garcia et al., 2012b)

**PCR and cloning experiments**

Six PCR primers were used to amplify products, assuming some linkage of 35S and 5S rRNA genes in either direct (head-to-tail) or inverted (tail-to-tail) orientations for several candidate species. The positions of the PCR primers are depicted in Supplementary Figure S2a. The primers used were: 5SgF (5’-GGTTGCACATCATTACGACT-3’) and 5SR (5’-GGTGCAACACAGAAGCTTC-3’) (Garcia et al., 2012a); 26SF (5’-AGAAGACTTAAAATACGGCAG-3’) and 18SR (5’-GGTTATCATTGGAGACAA-3’) are slightly modified versions of the Pr1 and Pr2 primers, respectively (Komarova et al., 2004); 26SR (5’-CTTTTCTCGGCCATTATTGATGC-3’) and 18SF (5’-GGGCATAC TGATCATTTCAACAGA-3’) primers are from (Kovarík et al., 2005). The variants of 5S genic primers were designed based on Ginkgo sequence:

- SgGFnF (5’-CAATGTTGCGATCTAC-3’) and SgGFnR (5’-GTGCAACTGGTGACCTTC-3’). Most reactions were conducted with DyNAzyme DNA polymerase (Finzymes, Espoo, Finland) or the PfuUltraTM II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA, USA).

To isolate IGS sequences we used PCR products obtained from reactions involving different combinations of primers. Multiple bands of variable sizes (from several hundred bp up to more than 2 kb) were obtained. The long fragments (>2 kb) were isolated using the QiAquick PCR purification kit (Hilden, Germany) and cloned into the pDrive vector (Qiagen PCR Cloning kit, Hilden, Germany). The PCR products obtained from PfuUltraTM II Fusion HS DNA Polymerase were treated with DyNAzymeII DNA Polymerase in the presence of dATP to add A overhangs. Several positive clones were recovered from each transformation reaction and analysed for insert length by gel electrophoresis. The IGS constructs were obtained for G. biloba (clone pGII2, containing linked 26S–IGS1–5S sequences, GenBank JX402064).

Restriction mapping was done using NEBcutter at the NEB server (http://tools.neb.com/NEBcutter2; Vincze et al., 2003). Restriction mapping was done using NEBcutter at the NEB server (http://tools.neb.com/NEBcutter2; Vincze et al., 2003). Searches for repeats were carried out by the tandem repeat finder program (Benson, 1999).

Subrepeated structure of IGS was analysed by matrix dot plot comparisons of using similarity search tools box at the Colorado State University (http://www.vivo.colostate.edu/molkit/dnadot/ and http://bioinfo.lifl.fr/yass/) (Noe and Kucherov, 2005). Sliding Windows were set to 9 with stringencies corresponding to 0–2 mismatches. Sequence divergences were documented (Ultralum, Claremont, CA, USA) and the PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide and photo documented (Ultralum, Claremont, CA, USA).

Bioinformatic analysis

The contigs of IGS clones were assembled in BioEdit Sequence Alignment Editor 7.0.9.0 (Hall, 1999) and blasted against the GenBank database. The rRNA coding sequences were identified by pairwise comparisons using dot plot graphical outputs (http://www.vivo.colostate.edu/molkit/dnadot/ and http://bioinfo.lifl.fr/yass/) (Nae and Kuchero, 2005). Sliding Windows were set to 9 with stringencies corresponding to 0–1 mismatches. Annotation of regulatory motifs was carried out based on analogy to A. thaliana gene (Choit et al., 2003). Restriction mapping was done using NEBcutter at the NEB server (http://tools.neb.com/NEBcutter2; Vincze et al., 2003). Searches for repeats were performed using the tandem repeat finder program (Benson, 1999). Subrepeated structure of IGS was analysed by matrix dot plot comparisons of using similarity search tools box at the Colorado State University (http://www.vivo.colostate.edu/molkit/dnadot/). Sliding Windows were set to 9 with stringencies corresponding to 0–2 mismatches. Sequence divergences were calculated by a DNADIST algorithm (Felsenstein, 1989). Phylogenetic trees were constructed using a SEAVIEW software (Gouy et al., 2010).

**RESULTS**

The data on fundamental rDNA organisation are provided here for 27 species, 21 genera, 4 families and 1 division of gymnosperms previously unstudied from this point of view. This provides coverage of basic rDNA organisation for at least one representative of all gymnosperm orders, as well as a 15% increase in coverage of genera and an almost 30% increase in coverage of families of gymnosperms. The newly assessed taxa are indicated in Table 2.
Table 2 List of taxa studied

| Order-family | Species |
|--------------|---------|
| Coniferales (Pinophyta) | |
| Pinaceae | Abies pinsapo Boiss. a |
| Coniferales (non-Pinophyta) | |
| Araucariaceae b | Araucaria bidwillii Hook.f |
| Cupressaceae | Cupressus macrocarpa Mast. b |
| Podocarpaceae | Podocarpus neriifolius (
| Taxaceae | Taxus baccata L. b |
| Cycadaceae | Cycas revoluta Thunb. b |
| Zamiaceae | Euphorbiaceae |
| Ginkgoales | Ginkgo biloba L. d |
| Gnetales a | Ephedra nebrodensis Tino b |
| Ephedraceae b | Ephedra viridis Cowl. e |
| Gnetaceae | Gnetum gnemon a |

All specimens were collected, except otherwise indicated, at the Barcelona Botanical Garden (Jardí Botànic de Barcelona) by Sílvia García, Samuel Pyke (curator of the Garden) and Miquel Vey (curator of the collections at the Botanical Institute of Barcelona). XXI 2011.

aNewly assessed division, family or species.
bNewly assessed genus and species.
cCollected at the Pedralbes Palace (Barcelona) by S Garcia (XI-2011).
dPlants grown at the greenhouse of the Botanical Institute of Barcelona (IBB).
eCollected at the Pius XXIII Square gardening (Barcelona) by S Garcia (XI-2011).
fCollected at the public Gardens of the Pedralbes Palace (Barcelona) by S Garcia (XI-2011).

Southern blot hybridisation reveals both S- and L-type arrangements in gymnosperms

The BamHI hybridisation profiles obtained for 25 species are shown in Figures 2 and 3. Basically, two hybridisation profiles could be distinguished:

- The first group (Figure 2) included species with profiles typical for a dominant linked arrangement (L-type) of both genes as revealed by extensive cohybridisation of 26S and 5S probes on blots. The four Podocarpus and the single Afrocarpus (both Coniferales) species showed multiple 5S and 26S cohybridising bands of 5–20 kb in size. Afrocarpus henkelii, P. elongatus, P. nerifolius and Afrocarpus falcatus (Podocarpaceae) had a similar restriction profile with respect to position and number of hybridisation bands (data not shown for P. nerifolius).

- Both Ephedra viridis and E. nebrodensis (Gnetales) had similar restriction profiles with all fragments hybridising to both 5S and 26S probes although in this case significant smearing of signals occurred.

There was a significant fraction of unresolved signals in >20 kb regions, which may indicate mutation of heavy methylation of units, as BamHI is sensitive to cytosine methylation (McClelland and Nelson, 1985). We therefore used EcoRI, which is less sensitive to methylation, to confirm the results (Supplementary Figure S3). The 26S and 5S probes cohybridised to one or two fragments in all species. Cohybridisation of fragments with all three rDNA (5S, 18S and 26S) probes was visible in A. falcatius, P. latifolia and both Ephedra species.

The BamHI hybridisation profile of Ginkgo (Figure 2) was somewhat different from those of Podocarpus, Afrocarpus and Ephedra. In Ginkgo, only high (>15 kb) molecular weight BamHI fragments cohybridised with the 26S and 5S probes while the low (6 kb) molecular weight fragment hybridised with the 26S probe only; however, the spacer probe, isolated from the pGIN2 clone (described further below) hybridised to both 6- and >15-kb fragments.

The second group (Figure 3) comprised species with a separated organisation of 5S and 35S repeats (S-type). In this group, the 5S probe hybridised to ladders of regularly spaced fragments of variable lengths considered to be indicative of tandem arrangement of units (Campbell et al., 1992). The 26S probe usually hybridised to one or several high-molecular-weight bands. The 5S hybridisation bands had no significant overlaps with the 26S signals except for a faint 4-kb fragment in Araucaria bidwillii (Araucariaceae, Coniferales). The periodicity of BamHI bands varied significantly from species to species ranging from 0.5 kb (Abies pinsapo) up to 1.8 kb in A. bidwillii. Relatively irregular ladders in Macrozamia moorei and Encephalartos mssinganus (both from Zamiaceae, Cycadaceae) may be explained by the presence of multiple BamHI sites in the unit monomer and/or more complex organisation of genes.

rDNA repeat size

In most gymnosperms analysed the rDNA probes hybridised to much larger restriction fragments than in angiosperms. We therefore wished to determine an approximate size of rDNA units of studied species. GenBank searches of 26S genes revealed that there is a single BglII site in the coding region (26S gene) that is highly conserved in all land plants. Consequently, digestion of genomic DNA with BglII would liberate monomeric units that could be revealed by subsequent hybridisation with the 5S, 18S and 26S probes (Supplementary Figure S3). Indeed, in all L-type species analysed (Podocarpus, Afrocarpus, Ephedra and Ginkgo), the probes hybridised to the high molecular weight fragments of >20 kb in length indicating that the size of basic 35S–5S units was within that range. The differences in the mobility of major BglII bands between species were only marginal. Fragments of smaller size (around 12 kb) seen in P. latifolia could be explained by the presence of additional BglII site(s) in some units. The sequence information on the Ginkgo rDNA unit allowed a more accurate estimation of unit size in this species. The genomic DNA was digested with the methylation-insensitive EcoRV, for which there are conserved target sites in the IGS1, IGS2 and 5.8S genes (Supplementary Figure S4). The subsequent hybridisation with the 5S, 18S and 26S probes revealed distinct fragments of 12, 3 and 6 kb, respectively, indicating that the size of the Ginkgo unit is 21 kb, at least.

Cloning of 35S–5S units from G. biloba

In G. biloba, the 26SF and 5SgR primers yielded several >2-kb PCR bands while the 26SF and 5SgF yielded a low molecular weight <1 kb band (Supplementary Figure S2). Cloning and sequencing of the 26S– 5S PCR products revealed that only high molecular weight 26SF/5SgR bands contained both 26S and 5S sequences while the low molecular
weight band amplified by the 26SF/5SgF primer set lacked either one or both genes and appeared to be non-specific. The inserts of five plasmid clones carrying the putative 26S–IGS1–5S sequences were slightly heterogeneous in size (1.6–1.9 kb). One clone carrying the 1827-bp insert (pGIN2 construct) was fully sequenced and submitted to GenBank (JX402064). BLAST searches revealed that there was a 5S gene ~1.6-kb downstream from the 26S gene (Figure 4). The 5S coding strand was the same as the one coding for the 26S gene (direct orientation). In the 120-bp coding region, the BamHI site was conserved, as were the regulatory motifs, Boxes A and C and the internal element. The upstream TATA sequence in the non-coding part at -33 was equally conserved. The pyrimidine-rich motif was found at the 5' end of the IGS1 (spacer between the 26S and 5S genes) as in other plant IGS sequences. The self-to-self alignment revealed the presence of two repetitive subregions on a dot plot (Supplementary Figure S5). The A1 subregion located 85–330-bp downstream from the 26S gene was composed of short 39-bp long units. The second subregion (A2) corresponding to the A subregion reported by Galión et al. (2012) was located within 671–1228 bp and contained 2.3 copies of a 240 bp repeat. The A2 monomeric unit was internally repetitive composed of several subrepeats of 39–57 bp in size. One subrepeat type was partially homologous to the A1 subrepeat. Sequence comparison with the 6-kb IGS clone (accession no. JQ279501.1) isolated by Galión et al. (2012) showed high identity over the 26S–IGS1–5S region, and differences were caused mostly by variation in the A1 and A2 tandem repeats. The sequences downstream from the 5S gene (IGS2 region) could not be compared because the pGIN2 clone lacked these sequences due to the cloning strategy. The pGIN2 clone also displayed significant similarities to non-5S bearing rDNA clones from Ginkgo at GenBank (Supplementary Figure S6).

**Cloning of 35S–5S units from Podocarpus elongatus**

In Podocarpus elongatus, the PCR using 5SgR/18SR and 5SgF/26SF yielded one prominent plus several minor bands (Supplementary Figure S2). The major 1.2-kb band from the 5SgF/26SF PCR reaction appeared to be non-specific as the DNA did not hybridise with the labelled 5S probe whereas the the 3-kb band obtained from the 5SgR/18SR reaction strongly hybridised with the probe (not shown). The 3-kb band was cloned and sequenced. The insert of clone p3g (GenBank JX402063) was 2612-bp long and contained part of the 5S and 18S genes on both the ends (Figure 4). The 5S was encoded by the opposite strand to the 18S gene (inverse orientation). The regulatory boxes A and C as well as the upstream regulatory elements were fully conserved. However, no internal element was found and there were also several point mutations, including the one in the BamHI site at the 5' end. The TATA regulatory element in the upstream non-coding region was not conserved. The IGS between the 5S and 18S genes (IGS2) was highly repetitive. The self-to-self alignment revealed the presence of two prominent repetitive subregions on a dot plot graph.
The B subregion at position 490–745 (numbers are from the first 5S nucleotide) was composed of short 28-bp subrepeats. The C subregion at position 1669–2391 was composed of 2.8 copies of a 257-bp repeat. The 18S coding sequence started at position 2580. A TATAGGG motif resembling the TATA(R)-TA(n)GGG transcription initiation site (Perry and Palukaitis, 1990) was located immediately upstream of the C subregion.

Cloning of 35S–5S units from *E. nebrodensis*

In *E. nebrodensis*, the PCR using 5SgGINF and 18SR yielded a single strong ~4 kb band plus several minor bands of larger size. The ~4 kb fragment was cloned and one clone (pEPHN4) was sequenced (GenBank JX843794). The insert was 3358 bp long and contained three 5S copies on one end and a 5′ terminus of the 18S gene on the other end. The 5S insertion formed a short tandem of closely (9 bp) spaced genes (Figure 4). The 5S rRNA was encoded by the same DNA strand as the 35S genes (direct orientation). The genic region of the first copy (5S1) was 100% identical to the sequence of 5S rRNA from *E. kokanica* (GenBank X06996 from Melekhovets et al. (1988)) with conserved regulatory elements. The second (Ψ5S2) and third (Ψ5S3) genes had a 40 bp deletion at the 5′ end and contained several mutations in regulatory boxes. Hence, these copies probably represent pseudogenes. The homology between pseudogenes and apparently functional 5S1 was 80–85% while that between both pseudogenes was 70% (Supplementary Figure S7). The terminators following coding region were, however, conserved in all the three units. Sequence downstream from the 5S genes was highly repetitive composed from short subrepeats (Supplementary Figure S5). Except of rRNA coding regions there was no significant homology between IGS clones from *Ginkgo*, *Podocarpus*, *Ephedra* and *Gnetum* (EMBL FR695703 from Wicke et al. (2011)).

**DISCUSSION**

Genomic organisation of vital rRNA genes has been a topic of numerous investigations in a wide range of biological taxa. Gymnosperms are considered as early diverging seed plants, and hence, represent an important transition between seedless plants and angiosperms. Here, we report on arrangement of 5S and 35S rDNA that may be linked (L-type) or separate (S-type) across the main gymnosperm orders.

Uniform S-type rDNA arrangement in Pinaceae (Coniferales) and Cycadales

A good deal of previous molecular cytogenetic studies had been focused on family Pinaceae, particularly on economically and ecologically important genera such as *Pinus* and *Picea*, among others. According to the cytogenetic and molecular data compiled (Table 1), the most commonly found rDNA arrangement was the unlinked (L-type) configuration. Nevertheless, partially overlapping 5S and 35S fluorescence *in situ* hybridisation signals were observed on chromosomes of *Abies alba* (Puizina et al., 2008), some *Picea* (Lubaretz et al.,...
1996; Brown and Carlson, 1997; Siljak-Yakovlev et al., 2002; Shibata and Hizume, 2008) or certain Pinus (Hizume et al., 2002; Cai et al., 2006; Islam-Faridi et al., 2007) species. However, the Southern blot hybridisation did not reveal significant cohybridisation of 26S (18S) and 5S probes in any of these, indicating that the most likely interpretation of colocalised signals is the juxtaposition of independent 5S and 35S arrays. The Pinaceae karyotypes typically display many more 35S than 5S loci (more than twice in most cases; Siljak-Yakovlev et al., 2002; Islam-Faridi et al., 2007). Yet, Southern hybridisations did not reveal extraordinary heterogeneity of signals (Figure 3b), which could be explained by interlocus homogenisation and/or recent origin of rDNA loci. On the other hand, length heterogeneity of 5S units is indicated in *Abies pinsapo*, consistent with previous studies in this genus (Besendorfer et al., 2005).

Species from the family Zamiaceae (Cycadales) investigated—genera *Ceratozamia*, *Zamia* and *Encephalartos*—clearly displayed an S-type rDNA pattern as in situ hybridisation data had yet pointed out for both *Ceratozamia* and *Zamia* (Tagashira and Kondo, 2001), the only genera of this group investigated to date. The fact that recent molecular phylogenetic approaches (Supplementary Figure S1) point towards cycadophytes being more ancient than *Ginkgo* (Mathews, 2009) hinders even more the interpretation about the ancestral condition of rDNA organisation in seed plants.

**Both S- and L-type rDNAs in non-Pinaceae Coniferales**

In this group, we observed certain variations in rDNA organisation. Most members of Araucariaceae, Cupressaceae and Taxaceae showed separation of both 5S and 35S loci. The 5S genes were organised in

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**Figure 4** Schematic representation 35S–5S units in *G. biloba*, *Podocarpus elongatus* and *E. nebrodensis*. Arrows above boxes indicate direction of transcription. The lines with arrows represent clones carrying 5S insertions. Subrepeated portions of IGS are indicated and labelled as (a–c). The 5S coding region sequences are shown below each graph, with conserved regulatory elements underlined. The positions of BamHI sites are indicated; in *Podocarpus* the site is mutated (asterisk).
long tandems as separate arrays. Araucaria bidwillii was interesting in having an extraordinarily long 3S–5S spacer (1.8 kb), which is much longer than that of any gymnosperm 5S spacers, whose units range between 0.4–0.87 kb (Gorman et al., 1992; Moran et al., 1992; Trontin et al., 1999; Besendorfer et al., 2005); known angiosperm 5S units range between 0.2–0.9 kb (Sastri et al., 1992; Fulnee et al., 2006). It is possible that the large size of IGS was caused by the expansion of repeated elements as apparently happened in Larix species (Trontin et al., 1999) or in A. alba (Besendorfer et al., 2005). It will be interesting to analyse positions of 5S genes on Araucaria chromosomes that appear to bear a single 35S locus (Miranda et al., 2007).

Of note is that the genera Podocarpus and the closely related Afrocarpus (Podocarpaceae) appear to be exceptional among non-Pinaceae conifers in showing clear linkage of 26S and 5S units. The 35S–5S arrays are highly homogeneous, without any evidence of abundant separate loci. The 5S insertions occurred between 6–9 kb from the 26S gene (estimates are based on Southern blot mapping) and >2-kb upstream of 18S gene (based on cloning). The cloned 5S–18S fragment could potentially transcribe both 5S and 35S genes as regulatory motifs for both RNA polymerases were identified in the sequence. The direction of transcription would be opposite as the rRNAs are encoded from complementary DNA strands. In a cytogenetic study, five Podocarpus species of Australian and New Zealand origin showed colocalisation of 35S and 35S signals, mostly on a single chromosome (Murray et al., 2002). In our study, four Podocarpus species of South African origin and one of the closely related genus Afrocarpus, showed linkage between 5S and 35S genes. Thus, it seems that the linked character of 5S and 35S genes is a characteristic feature for both genera that may comprise about 100 (Podocarpus) and up to 6 (Afrocarpus) species, respectively.

L- and S-type rDNAs in Gnetales

In this restricted group of plants, which in our sample consists only on both Ephedra viridis and E. nebrodensis (Ephedraceae) we found cohybridisation of 26S, 18S and 5S probes. Sequencing of the 5S–18S E. nebrodensis clone showed the presence of a functional 5S gene in a direct orientation as in Ginkgo. Contrast to Ginkgo, the E. nebrodensis clone contained additional pseudogenised 5S copies, which were directly linked to the functional copy. The phylogenetic relationships between 5S sequences showed that Ephedra genes are closely related to those of Pinaceae (in accord with the gnepine hypothesis—further below) while both of them are clearly separated from those of Pinaceae (in accord with the gnepine hypothesis). These observations would partially support the gnepine hypothesis although preliminary comparisons of 5S sequence divergence suggest an even more unique position of Gnetales in seed plant phylogeny (Garcia et al., unpublished results).

Homogenised L-type rDNAs in Ginkgoales

Opinions split on the organisation of 35S and 5S genes in G. biloba, only the representative of its order. While fluorescence in situ hybridisation showed colocalisation of 35S and 5S genes on chromosomes (Nakao et al., 2005; Galian et al., 2012), the PCR-cloning analysis of Wicke et al. (2011) failed to demonstrate 5S linkage to the large rDNA cluster. The pGIN2 clone isolated by us harboured a 5S insertion in direct orientation located downstream from the 26S gene. The different orientation of 5S insertions in Ginkgo and Podocarpus suggests their independent origins. The 5S coding region was 100% identical to the directly sequenced 5S rRNA from Ginkgo (GenBank M10433.1 from Horv et al., 1985) indicating that we cloned an apparently functional gene that is likely to be expressed. On the other hand, there were two mutations in the 5S coding region of the clone obtained by Galian et al. (2012) (GenBank JQ279501.1) although these may not influence gene functionality as both locate outside of the internal controlling region.

Despite these observations some uncertainty remains over the organisation of rDNA units in Ginkgo. This is because there have been six IGS clones obtained in different laboratories out of which only two (GenBank JQ279501 and JX402064, from the University of Valencia, ES and Academy of Sciences, CZ, respectively) contained 5S insertions, while these were absent in the remaining four clones (GenBank FR695705-7 and JQ279502, from the University of Vienna, AU and University of Valencia, ES, respectively). Furthermore, there is apparent inconsistency between the unit size calculated from cloning experiments (<12 kb, in Galian et al. 2012) and Southern blot mapping (>20 kb). So, we are left with several possible explanations. The clone to clone differences can be explained by un-homogenised rDNA pools containing multiple gene families and pseudogenised copies, as suggested by Galian et al. (2012). Next generation sequencing of ITS pools revealed that the intragenomic diversity of rDNA units is higher in species with multiple chromosomal loci than in species with a single locus in Nicotiana (Matyssek et al., 2012). In this context, sex chromosomes in G. biloba (Newcomer, 1954) distinguished by number of satellites that likely represent rDNA loci, may generate some unit diversity. However, the Southern hybridisations do not support significant heterogeneity in the spectrum of rDNA families, and >90% of rDNAs seem to form a single or possibly two families. Such pattern is consistent with clustered rDNA-fluorescence in situ hybridisation signals on Ginkgo chromosomes.
(Nakao et al., 2005; Galián et al., 2012). We favour the explanation that the differences between individual IGS clones (with and without 5S insertions) isolated in different laboratories could be caused by biased amplification of rare rDNA variants (pseudogenes) and/or a PCR failure to accurately amplify DNA templates. The latter hypothesis is supported by the observation that different thermostable polymerases appear to produce products of different lengths on Ginkgo’s DNA (not shown). The longest products were obtained with polymerases bearing a proofreading activity (3′→5′ exonuclease activity), and we recommend preferential usage of these polymerases when amplifying difficult IGS templates, in general.

Together, the data in this and others work (Galián et al., 2012) support the hypothesis that the predominant organisation of Ginkgo rDNAs is L-type.

**Comparison of rDNA organisation in gymnosperms and angiosperms**

The S-type arrangement seems to predominate in most angiosperm plant lineages (Campell et al., 1992; Sastrí et al., 1992; Wicke et al., 2011) while in some families the L-type arrangement is quite frequent (Garcia et al., 2010). In gymnosperms the situation may be similar, in that the species from the group with the greatest diversity (Pinaceae conifers, accounting for almost one quarter of gymnosperm diversity) have the separate arrangement; in other lineages, however, such as Podocarpaceae, Ephedraceae and Ginkgoaceae, the L-type arrangement seems to be the dominant. The phylogenetic relationships (Figure 1) indicate that the 5S rDNA has changed its fundamental genomic organisation at least three times during the evolution of gymnosperms. Hence, conclusions based on single species observations should not be generalised. As in the angiosperms, parallel existence of comparable number of L-type and S-type units in the same genome has not been observed, suggesting that homogenisation pressures acting on rDNA repeats are strong. Yet coexistence of minor S-type loci along with major L-type arrangement (and vice versa) cannot be excluded as in some angiosperm species (Garcia et al., 2010, 2012a).

Sequence analysis of 5S insertions, however, did reveal differences between angiosperms and gymnosperms. First, while in angiosperms the 5S insertions occur in a non-repetitive part of the IGS (within <1 kb downstream from the 26S gene), in gymnosperms the 5S gene is located distally to the 26S and 18S genes, embedded in a highly repetitive DNA region as in bryophytes (Sone et al., 1999). Second, to our knowledge the 5S genes of angiosperms are exclusively encoded by the opposite DNA strand to the 35S genes while in gymnosperms both direct and inverse orientations occur. Third, many angiosperm species bear retroelement signatures in regions flanking the 5S insertions resembling Cassandra TRIM elements (Kalander et al., 2008). No such features have been found in sequences of gymnosperm units. Perhaps, retroelement motifs might have already been eroded and/or replaced by tandem repeats in gymnosperms.

**Unit arrangement and chromosomal position of rDNA arrays**

Contrast to angiosperms, relatively little data exists on the chromosomal organisation of rDNA in gymnosperms. In most angiosperm species the 35S rDNA genes are located at (sub)telomeric positions of short arms (Roa and Guerra, 2012) while this trend is not so obvious in gymnosperms, in which interstitial positions were reported, particularly in Pinaceae (Islam-Faridi et al., 2007). However, in both gymnosperms and angiosperms analysed to date the L-type rDNAs occur at terminal position (Garcia et al., 2012b and García et al., unpublished). It will be interesting to determine chromosomal position of rDNA in other species in order to see whether the (sub)telomeric locations are favoured by the L-type arrays. It is also an open question of whether inversions, fusions (Olson and Gorelick, 2011) and other fundamental changes in chromosome structure (Leitch and Leitch, 2012) that accompany the long (300 Myrs) history of gymnosperm evolution contribute to switches in rDNA arrangements.

**CONCLUSIONS**

Our data show that in G. biloba the 5S genes integrated into the 26S–18S spacer and are nearly homogenised across the genome (L-type arrangement). Similar arrangement has been found in genera Podocarpus, Afrocarpus (non-Pinaceae Coniferales or cupressophytes) and Ephedra (Gnetales) while other gymnosperm lineages, including the cycads evolved a mostly separated arrangement of rDNAs. As both cycads and Ginkgo are considered to be early diverging 250–300 Myrs old species (Zhou and Zheng, 2003) and display contrasting L- and S-type arrangements, respectively, it is likely that the two rDNA organisations are evolutionary neutral as both are also present in modern plants. Rapid switches between fundamental organisations of these vital rRNA genes are unexpected, yet occurred frequently in both gymnosperm and angiosperm lineages. The overwhelming prevalence of S-type arrangement in seed plants, however, still needs to be explained.

**DATA ARCHIVING**

Sequence data have been submitted to GenBank: accession numbers JX402063, JX402064 and JX843794. IGS and 5S sequences from G. biloba deposited in the Dryad repository: doi:10.5061/dryad.fq228

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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