

## Comparative cytogenetic analysis of the genomes of the model grass *Brachypodium distachyon* and its close relatives

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- **Background and Aims** *Brachypodium* is a small genus of temperate grasses that comprises 12–15 species. *Brachypodium distachyon* is now well established as a model species for temperate cereals and forage grasses. In contrast to *B. distachyon*, other members of the genus have been poorly investigated at the chromosome level or not at all.
- **Methods** Twenty accessions comprising six species and two subspecies of *Brachypodium* were analysed cytogenetically. Measurements of nuclear genome size were made by flow cytometry. Chromosomal localization of 18–5.8–25S rDNA and 5S rDNA loci was performed by dual-colour fluorescence *in situ* hybridization (FISH) on enzymatically digested root-tip meristematic cells. For comparative phylogenetic analyses genomic *in situ* hybridization (GISH) applied to somatic chromosome preparations was used.
- **Key Results** All *Brachypodium* species examined have rather small genomes and chromosomes. Their chromosome numbers and genome sizes vary from  $2n = 10$  and 0.631 pg/2C in *B. distachyon* to  $2n = 38$  and 2.57 pg/2C in *B. retusum*, respectively. Genotypes with 18 and 28 chromosomes were found among *B. pinnatum* accessions. GISH analysis revealed that *B. pinnatum* with 28 chromosomes is most likely an interspecific hybrid between *B. distachyon* ( $2n = 10$ ) and *B. pinnatum* ( $2n = 18$ ). Two other species, *B. phoenicoides* and *B. retusum*, are also allopolyploids and *B. distachyon* or a close relative seems to be one of their putative ancestral species. In chromosomes of all species examined the 45S rDNA loci are distally distributed whereas loci for 5S rDNA are pericentromeric.
- **Conclusions** The increasing significance of *B. distachyon* as a model grass emphasizes the need to understand the evolutionary relationships in the genus *Brachypodium* and to ensure consistency in the biological nomenclature of its species. Modern molecular cytogenetic techniques such as FISH and GISH are suitable for comparative phylogenetic analyses and may provide informative chromosome- and/or genome-specific landmarks.

**Key words:** 5S rDNA, 25S rDNA, *B. distachyon*, *Brachypodium*, FISH, flow cytometry, GISH, nuclear genome size, ribosomal RNA genes.

### INTRODUCTION

*Brachypodium* is a small genus of temperate grasses which comprises 12–15 species (Catalan *et al.*, 1995; Catalan and Olmstead, 2000). Most of the species are from Mediterranean and Eurosiberian areas. For example, *B. distachyon* and *B. retusum* are Mediterranean, *B. rupestre* is a European taxon, while *B. pinnatum* and *B. sylvaticum* are Eurasian species. Two other species, *B. arbuscula* and *B. mexicanum*, have been described; the first is endemic to the Canary Islands, whereas the second grows in Central and South America. There are also fragmentary reports regarding two species from Africa, namely *B. bolussi* and *B. flexum* (Catalan and Olmstead, 2000). Phylogenetic classification of the genus has been controversial but the results of a recent study place it within an individual tribe named Brachypodieae (Sharma, 1979). Early molecular analyses based on nucleolar and chloroplast DNA provided some insight into the relationships between *Brachypodium* species (Shi *et al.*, 1993; Hsiao *et al.*, 1995). *Brachypodium distachyon* is sister to all other species, and *B. mexicanum* is sister to the remaining species analysed, which were all rhizomatous perennials (*B. arbuscula*, *B. retusum*, *B. rupestre*,

*B. phoenicoides*, *B. pinnatum* and *B. sylvaticum*; Catalan and Olmstead, 2000).

Broader phylogenetic analyses based on chloroplast *ndhF* and internal transcribed spacer of the nuclear rDNA repeat comparisons have demonstrated that the genus *Brachypodium* emerged soon after the divergence of Pooideae from Oryzoideae (Catalan *et al.*, 1997). Importantly, this indicates that members of the genus are more closely related to the key temperate cereals and forage grasses ('core pooids': Triticeae, Aveneae, Poeae) than is rice (Draper *et al.*, 2001). That is one of the reasons why *B. distachyon* has recently been recognized as an emerging model system for both comparative and functional genomics in grass species (Huo *et al.*, 2009). Many other attractive biological and physiological features, such as small and compact genome, low number of chromosomes, short life cycle and inbreeding habit, make *B. distachyon* a promising and useful tool for genomic analysis of the much larger and more complex cereal genomes (Hasterok *et al.*, 2006c; Garvin *et al.*, 2008). In 2007 an international consortium began whole genome sequencing of *B. distachyon*, which is expected to be completed shortly (<http://www.brachypodium.org/>).

The genus *Brachypodium* consists of species with small genomes and low base chromosome number ( $x = 5, 7, 8$  and 9)

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(Robertson, 1981). Apart from the model species *B. distachyon*, members of the genus have so far been poorly characterized cytogenetically. Among different *B. distachyon* ecotypes Robertson (1981) observed individuals with three different somatic chromosome numbers (10, 20 and 30) and assumed that this species had evolved a polyploid series based upon  $2n = 2x = 10$ . However, more detailed comparative cytological analyses using fluorescence *in situ* hybridization (FISH) with total nuclear and ribosomal DNA as probes revealed an allotetraploid nature of all forms with  $2n = 30$  (Hasterok *et al.*, 2004). This has since been confirmed by heterologous landing of *B. distachyon*-derived BAC clones onto chromosomes of forms with 20 and 30 chromosomes (Hasterok *et al.*, 2006c). All these results suggest more complex evolution of the three *Brachypodium* cytotypes than initially anticipated. Therefore, it is interesting to investigate further the relationships between *B. distachyon* and other species of the genus. In this study six species and two subspecies of *Brachypodium* were analysed cytogenetically. These analyses used somatic metaphase chromosomes and multicolour FISH with repetitive DNA sequences, such as 5S rDNA and 25S rDNA. In addition, genomic *in situ* hybridization (GISH) was carried out and estimations of nuclear genome size were made by flow cytometry.

## MATERIALS AND METHODS

### Plant material

Six species of *Brachypodium* P. Beauv. and two subspecies comprising 20 accessions were analysed. Seeds were obtained from research centres and botanical gardens. Information on the origins of this plant material is provided in Table 1.

TABLE 1. Original identities, sources and origins, somatic chromosome numbers ( $2n$ ), total numbers of 5S and 25S rDNA sites (and the fraction of these sites at which the two types of rDNA are co-localized) and nuclear genome sizes of *Brachypodium* species used in this study

Species	Accession no.	Origin	Source*	$2n$	Number of rDNA sites			pg/2C DNA	s.d.
					5S	25S	(5S + 25S)		
<i>B. distachyon</i>	ABR1	Turkey	a	10	2	2	–	0.631	0.015
<i>B. phoenicoides</i>	PI 89817	Spain	b	28	4	4	–	1.476	0.049
	PI 253503	Spain	b	28	4	4	–	1.499	0.005
	PI 283196	Portugal	b	28	4	4	–	1.497	0.009
<i>B. pinnatum</i>	PI 230114	Iran	b	18	2	2–3	–	0.882	0.005
	PI 345982	Norway	b	18	2	2	–	0.881	0.027
	PI 4193	Germany	c	28	4	4	–	1.462	0.007
	PI 249722	Greece	b	28	4	4	–	1.547	0.064
	PI 251445	Turkey	b	28	4	4	–	1.574	0.038
	PI 430277	Ireland	b	28	4	4	–	1.532	0.037
<i>B. retusum</i>	PI 4195	Greece	c	38	6	6	(4)	2.570	0.036
<i>B. rupestre</i>	PI 4196	Greece	c	18	2	4	–	0.843	0.011
<i>B. rupestre</i> subsp. <i>rupestre</i>	PI 440172	Russia	b	18	2	2	–	0.747	0.009
<i>B. sylvaticum</i>	PI 2058	?	d	18	2	4	–	0.832	0.004
	PI 237792	Spain	b	18	2	4	–	0.863	0.029
	PI 269842	Turkey	b	18	2	5–6	–	0.863	0.029
	PI 297868	Australia	b	18	2	6	–	0.873	0.008
	PI 318962	Spain	b	18	2	5	–	0.844	0.007
	PI 380758	Iran	b	18	2	4	–	0.898	0.045
	PI 4202	Greece	c	16	2	2	–	0.886	0.012

\* a, Brachyomics, Aberystwyth University, UK; b, US Department of Agriculture, USA; c, Botanical Garden Berlin – Dahlem, Germany; d, Botanical Garden Göttingen, Germany.

### Somatic chromosome preparations

The preparations were made according to the protocol described in detail in Hasterok *et al.* (2006a). Briefly, dehusked seeds were germinated on filter paper moistened with tap water at 20–22 °C in the dark for 3–4 d. Whole seedlings with roots 1.5–2.5 cm long were immersed in ice-cold water for 24 h, fixed in 3:1 (v/v) methanol/glacial acetic acid at 4 °C for several hours, and then stored at –20 °C until use. Excised roots were washed in 0.01 M citric acid – sodium citrate buffer (pH 4.8) for 20 min and enzymatically digested in a mixture comprising 20% (v/v) pectinase (Sigma, St Louis, MO, USA), 1% (w/v) cellulase (Calbiochem, San Diego, CA, USA) and 1% (w/v) cellulase ‘Onozuka R-10’ (Serva, Heidelberg, Germany) for 2 h at 37 °C. Meristems were dissected out from root tips, squashed in drops of 45% acetic acid and frozen. After freezing, cover-slips were removed and the preparations were post-fixed in 3:1 ethanol/glacial acetic acid, dehydrated in absolute ethanol and air dried.

### DNA probes and FISH

The following DNA probes were used.

- (1) The 5S rDNA probe was obtained from the wheat clone pTa794 (Gerlach and Dyer, 1980) by PCR amplification and labelling with tetramethyl-rhodamine-5-dUTP (Roche, Indianapolis, IN, USA). The sequences of oligonucleotide primers and conditions for the reaction are as described by Hasterok *et al.* (2004).
- (2) The 25S rDNA probe was generated by nick translation of a 2.3-kb *Cla*I subclone of the 25S rDNA coding region of *Aradopsis thaliana* as described by Jenkins and Hasterok

(2007). This probe was labelled with digoxigenin-11-dUTP (Roche) and used to visualize 45S rDNA loci containing the genes coding for 18S, 5.8S and 25S rRNA.

- (3) For GISH experiments, a standard procedure of DNA isolation using CTAB (cetyltrimethylammonium bromide) was used. This method is described in detail in Murray and Thompson (1980). Non-sheared nuclear DNA of *B. distachyon*, *B. sylvaticum*, *B. rupestre* and *B. pinnatum* was labelled by nick translation either with digoxigenin-11-dUTP or with tetramethyl-rhodamine-5-dUTP.

The FISH procedure followed the protocol described in detail by Jenkins and Hasterok (2007). The general conditions were as follows: the hybridization mixture consisted *inter alia* of 50% deionized formamide, 20% dextran sulfate, 2× saline sodium citrate (SSC) and salmon sperm blocking DNA in 50–100× excess of labelled probes. All probes were mixed to a final concentration each of about 2.5 ng  $\mu\text{L}^{-1}$  and pre-denatured (75 °C for 10 min). The slides with chromosome material and the hybridization mixture were then denatured together at 70 °C for 4.5 min in an Omnislide *in situ* hybridization system (Hybaid, Basingstoke, UK) and allowed to hybridize for 12–20 h in a humid chamber at 37 °C. After hybridization, slides were washed in two changes of 10% deionized formamide in 0.1× SSC, for 5 min each, at 42 °C, which is equivalent to 79% stringency. Immunodetection of digoxigenated probes was performed according to standard protocols using fluorescence isothiocyanate (FITC)-conjugated anti-digoxigenin antibodies (Roche). The preparations were mounted and counterstained in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 2.5  $\mu\text{g mL}^{-1}$  4',6-diamidino-2-phenylindole (DAPI; Serva).

#### Image capturing and processing

All images were acquired using a Hamamatsu C5810 CCD camera attached to an Olympus Provis AX epifluorescence microscope. The images were processed uniformly and superimposed using Micrografx (Corel) Picture Publisher software.

#### Flow cytometry

For flow cytometry, suspensions of nuclei were prepared from leaves of young plants. The samples were prepared as described by Hajdera *et al.* (2003). Samples were measured using a DAKO Galaxy flow cytometer. The final DNA content for each *Brachypodium* accession was calculated on the basis of three individual measurements. The nuclear DNA content of *B. distachyon* was calculated against nuclei isolated from young leaves of *Brassica rapa* 'Goldball' (0.97 pg/2C DNA; D. Siwinska, University of Silesia, Katowice, unpubl. res.) as a standard. *Lycopersicon esculentum* 'Stupicke' (1.96 pg/2C DNA; Dolezel *et al.*, 1992) was used as a standard to measure DNA amounts in *B. phoenicoides*, *B. retusum* and four accessions (PI430277, PI251445, PI249722, 4193) of *B. pinnatum*. For two other accessions of *B. pinnatum* (PI345982, PI230114) and for *B. sylvaticum* and *B. rupestre*, *B. oleracea* 'Amager' (1.3 pg/2C DNA; D. Siwinska, unpubl. res.) was used as a standard. FloMax (Partec) software was used to process data.

## RESULTS AND DISCUSSION

### Chromosome number and genome size

Somatic chromosome numbers and nuclear DNA amounts (pg/2C DNA) were calculated for 20 accessions representing six *Brachypodium* species (Table 1). The lowest chromosome number was observed in *B. distachyon* ( $2n = 10$ ) and the highest in *B. retusum* ( $2n = 38$ ). All accessions of *B. rupestre* and *B. sylvaticum* analysed, with the only exception of *B. sylvaticum* subsp. *glaucovirgens* ( $2n = 16$ ), have 18 chromosomes. Unexpected results were obtained for *B. pinnatum* where two accessions with 18 chromosomes were found, and four with 28. The accessions of *B. pinnatum* with 28 chromosomes may be presumed to represent polyploid cytotypes. This may also be the case for another species with the same chromosome number, i.e. *B. phoenicoides*, and for *B. retusum* ( $2n = 38$ ).

*Brachypodium distachyon* has the smallest genome among the species examined, only 0.631 pg/2C DNA. The highest nuclear DNA content (2.57 pg/2C) was estimated for *B. retusum*. For the first time, nuclear DNA amount was measured for *B. rupestre* ( $2n = 18$ ), *B. rupestre* subsp. *rupestre* ( $2n = 18$ ), *B. sylvaticum* subsp. *glaucovirgens* ( $2n = 16$ ), *B. phoenicoides* ( $2n = 28$ ) and *B. retusum* ( $2n = 38$ ). The three species with 18 chromosomes (*B. pinnatum*, *B. rupestre* and *B. sylvaticum*) have similar sized nuclear genomes, ranging from 0.747 to 0.898 pg/2C DNA. The average content obtained for six accessions of *B. sylvaticum* was 0.862 pg/2C, and similar results (0.881 pg/2C) were seen for two *B. pinnatum* ( $2n = 18$ ) accessions. The average genome size for three accessions of *B. phoenicoides* was 1.491 pg/2C DNA, with a very similar average amount for four  $2n = 28$  chromosome forms of *B. pinnatum* (1.528 pg/2C). The RBG Kew Plant DNA C-values database (Bennett and Leitch, 2004; <http://www.kew.org/cvalues/homepage.html>) contains information about genome size for only three *Brachypodium* species: *B. distachyon* (0.72 pg/2C), *B. sylvaticum* (0.96 pg/2C) and *B. pinnatum* (2.46 pg/2C). The values obtained here for the first two are respectively 12.36 and 10.21% lower than those from the Kew database, while the one for *B. pinnatum* ( $2n = 28$ ) is approximately 37.9% lower than the one published in that database. Possible reasons for such differences include significant intraspecific DNA content variability, a phenomenon which was recently demonstrated for some other grass species, for example wild populations of *Festuca pallens* (Smarda *et al.*, 2008). However, such discrepancies are most likely caused by various methodological factors, for example different estimation methods of DNA contents and different standards used for genome size calculation (Dolezel and Bartos, 2005; Greilhuber, 2005, 2008).

### Distribution of rRNA genes

Most *Brachypodium* species have rather small chromosomes, which in many cases are similar in shape and size, and therefore are difficult to distinguish. Their lengths at mitotic metaphase after standard incubation of seedlings in ice-cold water ranged from approximately 2 to 6.5  $\mu\text{m}$ . Thus, additional landmarks are required to enable chromosome identification. Many

repetitive DNA sequences can be localized by FISH and provide markers useful for identification of individual chromosomes (Maluszynska, 2002). The comparison of chromosome number and distribution of rDNA loci can provide valuable information about the phylogenetic relationship between related taxa, for example in ornamental lilies (Marasek *et al.*, 2004), various Brassicaceae (Hasterok *et al.*, 2001, 2006b), *Hypochaeris* (Weiss-Schneeweiss *et al.*, 2003) and *Aloe* (Adams *et al.*, 2000) species.

To date, the number and localization of rDNA sites have been determined only for *B. distachyon* itself and for the former '*B. distachyon* forms' with 20 and 30 chromosomes (Hasterok *et al.*, 2004). Total numbers of 5S rDNA and 25S rDNA sites in all 20 accessions of the six species analysed in the present study are summarized in Table 1. The fractions

of sites at which the two classes of ribosomal RNA genes are co-localized are also indicated. Simultaneous FISH with 5S rDNA and 25S rDNA probes identified two pairs of chromosomes carrying one of each rDNA sequence in *B. distachyon* (Fig. 1A), *B. rupestre* subsp. *rupestre* (Fig. 1C) and *B. sylvaticum* subsp. *glaucovirens* (Fig. 1G) and in two accessions of *B. pinnatum*: PI345982 (Fig. 2A) and PI230114 (data not shown). The chromosomal distribution of 25S rDNA sites is always distal on the short arm, which occasionally may be extended into a distinct secondary constriction (Fig. 1G), while 5S rRNA sites always occupy proximal and pericentromeric parts of the relevant chromosome long arm. Genotypes of *B. rupestre* (Fig. 1B) and three *B. sylvaticum* accessions, PI237792 (Fig. 1D), PI380758 and PI2058 (data not shown), contain one pair of chromosomes bearing 5S rDNA and two

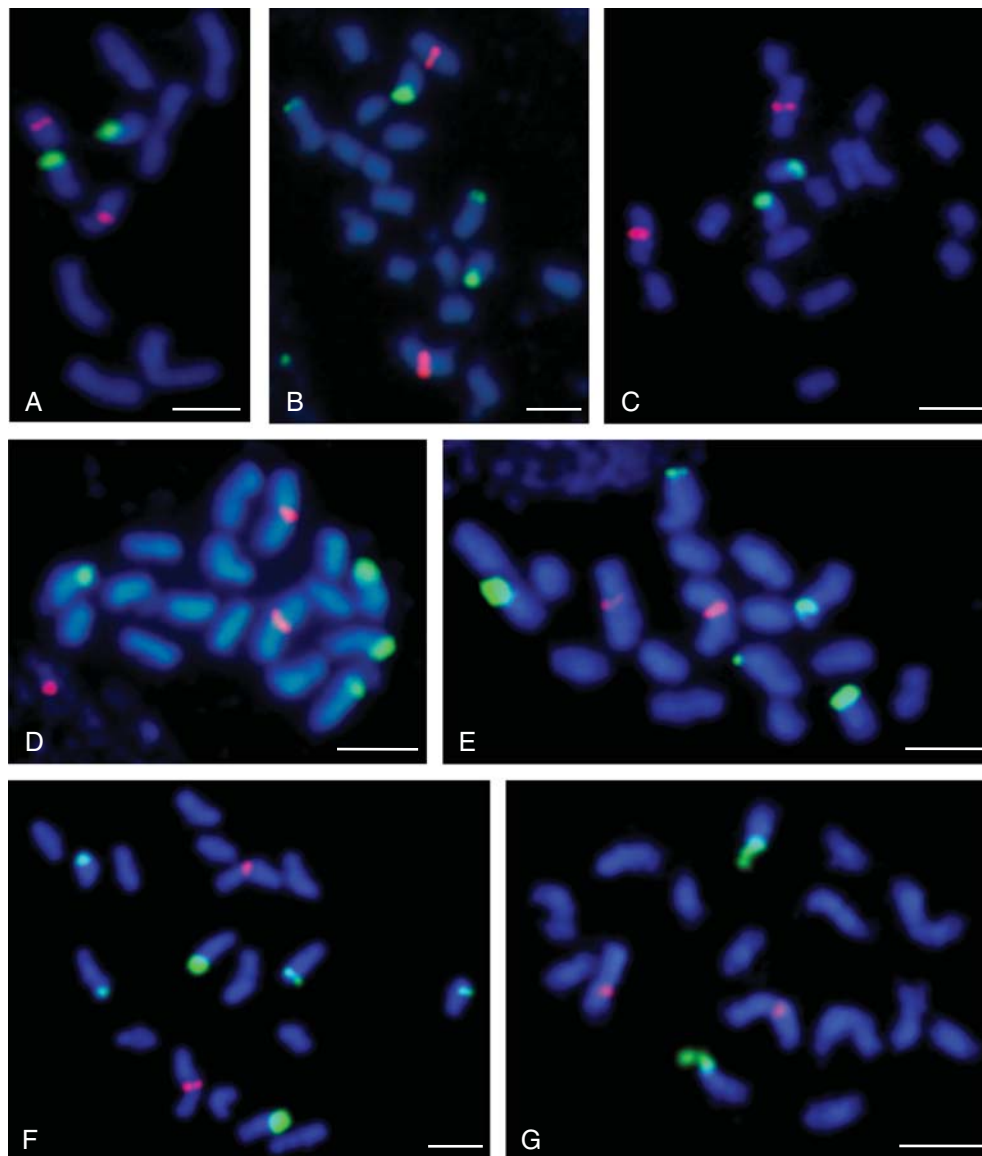


FIG. 1. Distribution of 5S (red) and 25S (green) ribosomal DNA sites determined by FISH to somatic metaphase chromosomes of (A) *Brachypodium distachyon*, (B) *B. rupestre*, (C) *B. rupestre* subsp. *rupestre*, (D) *B. sylvaticum* PI237792, (E) *B. sylvaticum* PI318962, (F) *B. sylvaticum* PI269842 and (G) *B. sylvaticum* subsp. *glaucovirens*. The chromosomes are counter-stained with DAPI (blue). Scale bars = 5 μm.

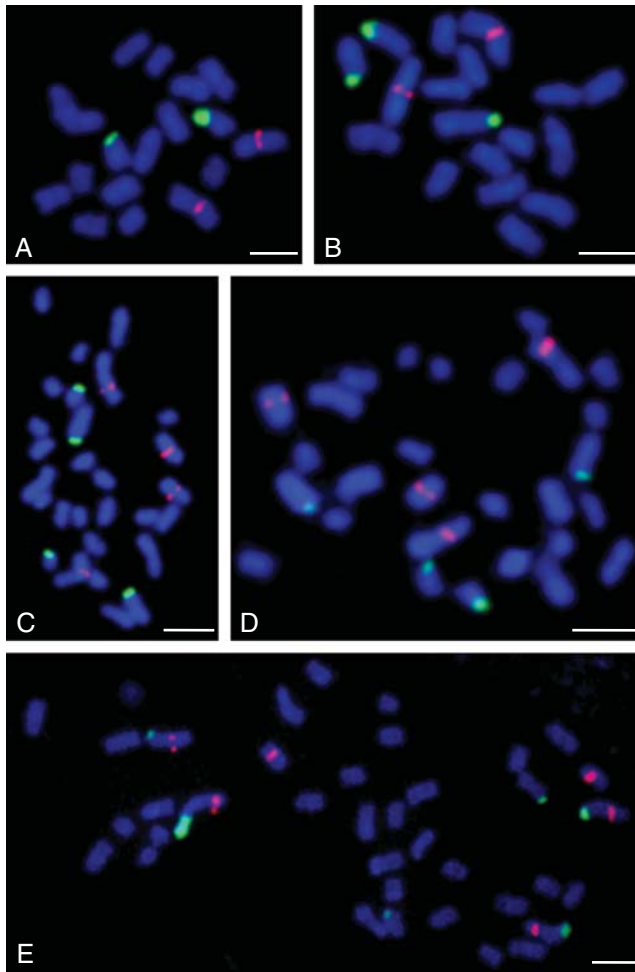


FIG. 2. FISH of 5S (red) and 25S (green) rDNA probes to chromosomes of: (A) *B. pinnatum* PI345982, (B) *B. pinnatum* PI230114, (C) *B. pinnatum* PI251445 ( $2n = 28$ ), (D) *B. phoenicoides* PI253503 and (E) *B. retusum*. Scale bars = 5  $\mu\text{m}$ .

other pairs with 25S rDNA sites. The number of 25S rDNA sites varies with different *B. sylvaticum* accessions. The lowest number is found in the three genotypes mentioned above, the intermediate, 5, in the genotype PI318962 (Fig. 1E) and in some individuals of the PI269842 genotype (data not shown) while the highest, 6, is seen in two other accessions of *B. sylvaticum*, PI269842 (Fig. 1F) and PI297868 (data not shown). Similar variation was also observed in one accession of *B. pinnatum* with 18 chromosomes, where in some individuals of PI230114 (Fig. 2B) three sites of 25S rDNA were observed. The phenomenon of numerical variation in 25S rDNA sites was *inter alia* also reported for some ecotypes of *A. thaliana* (Fransz *et al.*, 1998) and *Brassica* species (Hasterok *et al.*, 2006b). Such differences could be explained by chromosome rearrangements, unequal crossing-over or gene conversion (Dubcovsky and Dvorak, 1995; Lim *et al.*, 2000).

Accessions of *B. pinnatum* ( $2n = 28$ ) invariably have two pairs of chromosomes with distally localized 25S rRNA gene clusters and the same number of chromosomes with a pericentromeric distribution of 5S rDNA sites (Fig. 2C).

The two pairs of each type are morphologically distinct from each other, which supports the proposed allopolyploid origin of *B. pinnatum* with 28 chromosomes. The same number and distribution of rRNA gene sites was found in *B. phoenicoides* chromosomes (Fig. 2D). Another species analysed, *B. retusum*, exhibits the highest number and most diverse localization of rDNA, distributed in four chromosome pairs (Fig. 2E). Two pairs of chromosomes bear both 25S rRNA and 5S rRNA gene clusters, while the other two pairs carry only one type of rDNA each.

Cytogenetic analysis with rDNA shows that these genes are effective landmarks for only a few chromosomes in *Brachypodium* karyotypes but their chromosomal distribution is very conserved among all species studied. Similarly, poor differentiation in chromosomal organization of rDNA loci was observed in the genera *Glycine* (Singh *et al.*, 2001), *Lupinus* (Naganowska and Zielinska, 2002, 2004; Hajdera *et al.*, 2003) and *Setaria* (Benabdelmouna *et al.*, 2001). Despite the rather uniform chromosomal distribution of rDNA sites, comparison of their number and localization allows some inferences to be made about the ploidy level and nature of all *Brachypodium* species under analysis.

#### GISH for comparative analysis and phylogenetic studies

The allopolyploid status of some *Brachypodium* species has been verified by GISH. This variant of FISH is widely applied to identify entire parental genomes, individual chromosomes or their fragments in natural allopolyploids as well as in synthetic interspecific and intergenomic hybrids (Kim *et al.*, 2008; Kopecky *et al.*, 2008; Pendinen *et al.*, 2008; Molnar *et al.*, 2009). *In situ* hybridization of total nuclear DNA of one species to chromosomes of related species can provide valuable information about phylogenetic relationships and chromosomal rearrangements that occurred during the evolution of these species. Figure 3A shows the result of hybridization of *B. distachyon* nuclear DNA to chromosomes of this species. Signals are present on every chromosome of the complement with maximum intensity in the pericentromeric regions, whereas chromosome arms show much weaker and diffused fluorescence. The same result was obtained after hybridization of genomic DNA from *B. sylvaticum* to its own chromosomes (Fig. 4B). The uneven localization of hybridization signals most likely reflects the distribution of repetitive DNA along chromosomes. Various fractions of DNA that is detected by GISH and uneven distribution of hybridization signals has often been observed in other species with small and compact nuclear genomes, for example in *Medicago* (Falistocco *et al.*, 2002), *Brassica* (Maluszynska and Hasterok, 2005), *Arabidopsis* (Ali *et al.*, 2004) and *Oryza* (Li *et al.*, 2001a, b).

In this comparative study genomic DNA isolated from *B. distachyon* was hybridized to root-tip chromosomes of four other *Brachypodium* species, *B. sylvaticum*, *B. rupestre*, *B. pinnatum* ( $2n = 18$ ) and *B. retusum* (Fig. 3B–E, respectively), producing signals that were predominantly concentrated at pericentromeric locations. Noticeably, the signal intensity is not the same on all chromosomes of the complements. The *B. distachyon* probe labels strongly only two chromosomes

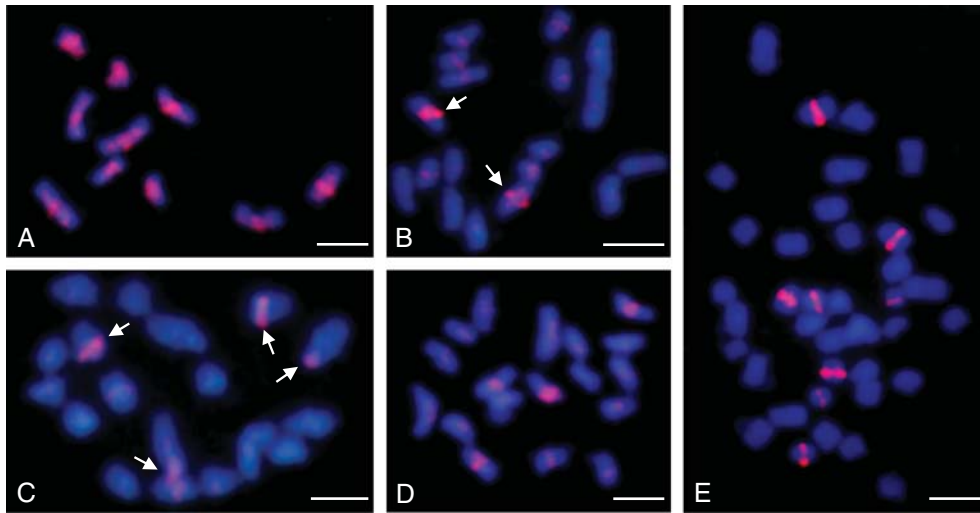


FIG. 3. Single-target GISH. Total nuclear DNA of *B. distachyon* (red) hybridizing with chromosomes of (A) *B. distachyon*, (B) *B. sylvaticum*, (C) *B. rupestre*, (D) *B. pinnatum* and (E) *B. retusum*. Arrows mark chromosomes with stronger hybridization of the genomic probe. Scale bars = 5  $\mu\text{m}$ .

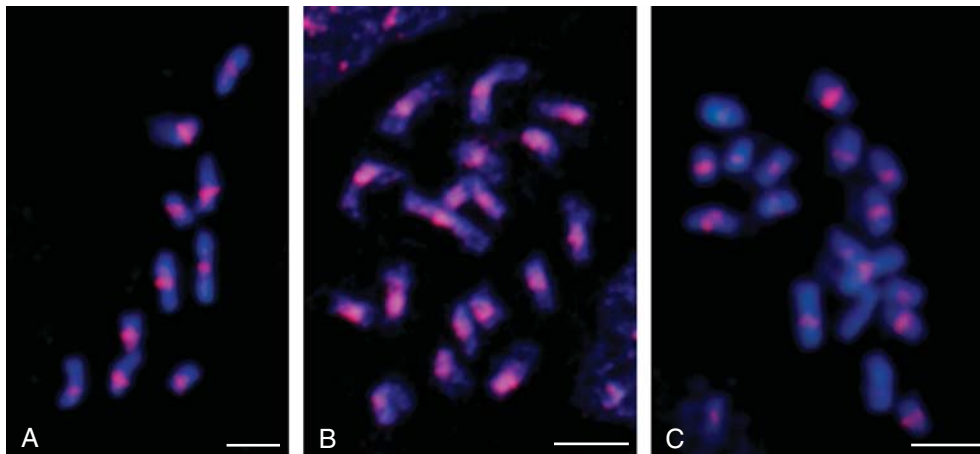


FIG. 4. GISH of *B. sylvaticum* genomic DNA to chromosomes of (A) *B. distachyon*, (B) *B. sylvaticum* and (C) *B. rupestre*. Scale bars = 5  $\mu\text{m}$ .

of *B. sylvaticum* (Fig. 3B; arrows), while in the complements of *B. rupestre* (Fig. 3C; arrows) and *B. pinnatum* ( $2n = 18$ ; Fig. 3D) four chromosomes with stronger fluorescence can be observed. It is probable that these signals coincide with the 5S rDNA sites in *B. sylvaticum* and with chromosomal distribution of both rDNA types in *B. rupestre*. Similar observations were reported for *Medicago* (Falistocco *et al.*, 2002) and *Brassica* (Maluszynska and Hasterok, 2005) genomes. In addition, Belyayev *et al.* (2001) demonstrated that total nuclear DNAs isolated from species representing several main groups of Poaceae revealed extensive conservation especially in pericentromeric regions and nucleolus organizing regions when hybridized with *Aegilops speltoides* chromosomes. In the present study, this conservation seems to be not only restricted to 45S rDNA but may also involve the regions occupied by 5S rRNA genes. Interestingly, two pairs of strong signals are observed in pericentromeric regions of *B. pinnatum* chromosomes (Fig. 3D), whereas there is only one pair of chromosomes known to bear 5S rDNA sites localized in such regions.

Contrary to the results obtained after hybridization with *B. distachyon* genomic DNA, GISH with *B. sylvaticum* nuclear DNA as a probe revealed signals in pericentromeric regions in the majority of *B. rupestre* (Fig. 4C) and *B. pinnatum* ( $2n = 18$ ) chromosomes (data not shown). Similar signals were also observed in all *B. distachyon* chromosomes (Fig. 4A). Such results may indicate that there are repetitive sequences common to all examined *Brachypodium* genomes while *B. distachyon* has some genome-specific repeats, seen in the experiment with total nuclear DNA of *B. distachyon* as a probe (Fig. 3). The high homology of repeats indicates closer phylogenetic relationships among *B. sylvaticum*, *B. rupestre* and *B. pinnatum* ( $2n = 18$ ) than between these species and *B. distachyon*, which is in agreement with the results of earlier molecular analyses (Shi *et al.*, 1993; Catalan *et al.*, 1995; Catalan and Olmstead, 2000).

Interestingly, hybridization of *B. distachyon* genomic DNA to *B. retusum* root-tip cells distinguished eight out of 38 chromosomes of the complement (Fig. 3E). This result

strongly supports the proposed allopolyploid origin of *B. retusum*, with *B. distachyon* appearing to be one of its putative ancestral species. The lower than expected number of

chromosomes carrying *B. distachyon* total nuclear DNA signals could be caused by the loss of two chromosomes originating from *B. distachyon* in the *B. retusum* complement. Alternatively, these chromosomes may be present but with different repeat composition in their pericentromeric regions. The same probe applied to 28 chromosomes of *B. pinnatum* lights up ten chromosomes, whereas in *B. phoenicoides* 12 chromosomes carry the signals (data not shown).

Another GISH experiment was done with differentially labelled nuclear DNAs of both *B. distachyon* (red fluorescence) and *B. sylvaticum* (green fluorescence) that were simultaneously used as probes to hybridize with chromosomes of *B. phoenicoides*. This experiment revealed clear signals of *B. sylvaticum* genomic probe in the pericentromeric regions of each *B. phoenicoides* chromosome of the complement. Twelve out of 28 chromosomes of this species hybridized also with *B. distachyon* nuclear DNA probe (Fig. 5A). A similar result was obtained after simultaneous hybridization

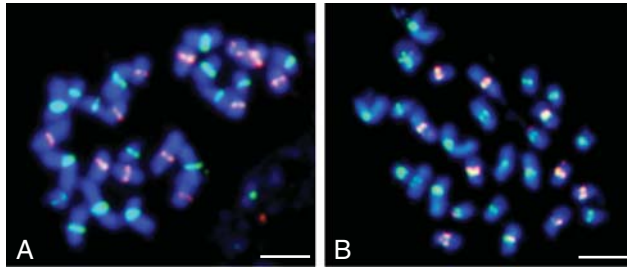


FIG. 5. Multicolour genomic *in situ* hybridisation. (A) Genomic DNA of *B. sylvaticum* (green) and *B. distachyon* (red) hybridizing to chromosomes of *B. phoenicoides*. (B) Genomic DNA of *B. pinnatum* 2n = 18 (green) and *B. distachyon* (red) hybridizing to metaphase chromosomes of *B. pinnatum* 2n = 28. Scale bars = 5 µm.

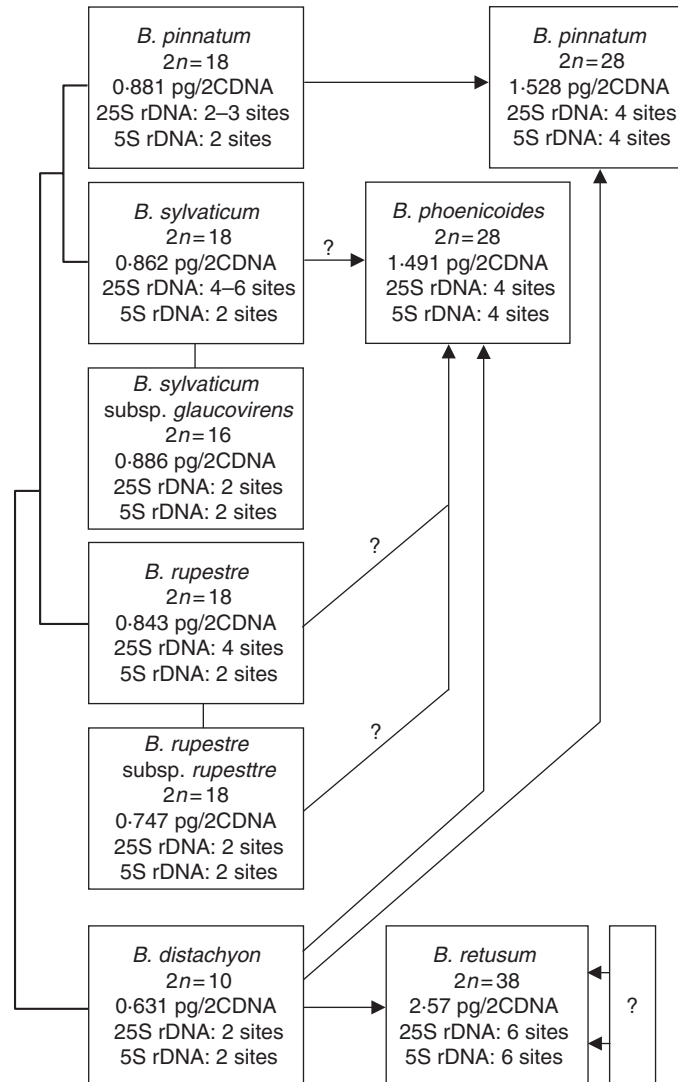


FIG. 6. Hypothetical relationships between *Brachypodium* species based on chromosome number, genome size, GISH results and rDNA loci mapping. Arrows show putative parental species for the polyploid (*B. pinnatum* 2n = 28, *B. phoenicoides*, *B. retusum*) species. Arrows with question marks indicate that *B. sylvaticum* and *B. rupestre* are equally likely to be one of the putative ancestral species of *B. phoenicoides*.

of *B. phoenicoides* chromosomes with *B. distachyon* and *B. rupestre* DNA (data not shown). These results suggest that *B. distachyon*, or a close relative, is one of the putative ancestors of *B. phoenicoides*. The origin of the second progenitor is unresolved, because of the high proportion of sequences in common among candidate species. A similar experiment was performed on *B. pinnatum* ( $2n = 28$ ). The number of chromosomes in its karyotype as well as the number and localization of rDNA sites (Fig. 2C) suggest that this species is also an allopolyploid. To determine the genome composition of *B. pinnatum* with 28 chromosomes several multicolour GISH experiments were performed. Simultaneous hybridization of *B. pinnatum* ( $2n = 18$ ; green fluorescence) and *B. distachyon* (red fluorescence) total nuclear DNAs to *B. pinnatum* ( $2n = 28$ ) revealed signals of the former probe to be present in pericentromeric regions of every chromosome in the complement. However, *B. distachyon* genomic DNA hybridized only to ten out of 28 chromosomes (Fig. 5B). Two other experiments where *B. sylvaticum* or *B. rupestre* nuclear DNAs, respectively, were used as the complement to *B. distachyon* genomic DNA-based probe failed to distinguish chromosomes that belong to putative ancestral genomes (data not shown). On the basis of these results it can be concluded that *B. pinnatum* with 28 chromosomes is an interspecific hybrid containing genomes identical or similar to those of *B. distachyon* and *B. pinnatum* ( $2n = 18$ ). Two other species of *Brachypodium*, *B. phoenicoides* and *B. retusum*, are also allopolyploids with *B. distachyon* or its close relative as one of the putative ancestral species. A summary of the interrelationships between *Brachypodium* species based upon the number of chromosomes, nuclear genome size, GISH results and the numbers of ribosomal DNA loci is shown in Fig. 6.

The rapidly increasing significance of *B. distachyon* as a model grass highlights the need to undertake a broad comparative study to clarify the genome organization of related species in the genus. It is important not only for better understanding of the origin and evolution of the model species but also to establish a consistent biological nomenclature of *Brachypodium* species and therefore to increase the credibility of the material stored in germplasm banks and seed collections. We believe that this study should help to reach both of these objectives. Currently, a wealth of tools and resources are becoming available for further cytogenetic analysis of *Brachypodium* genomes. *Brachypodium distachyon* genomic DNA BAC libraries (Hasterok *et al.*, 2006c; Huo *et al.*, 2006) have already proved to be invaluable (Jenkins and Hasterok, 2007), providing not only chromosome- but even arm- and region-specific probes that effectively hybridize also with chromosomes of other *Brachypodium* species, allowing complex, comparative experiments to be carried out (Hasterok *et al.*, 2006a). Detailed annotations of ‘supercontigs’ covering large regions of *B. distachyon* nuclear sequence, derived from ordered BAC libraries, will soon be available (<http://www.brachypodium.org/>). Consolidation of these tools and resources should allow a comparative chromosome painting approach to be developed similar to that which is already available for *Arabidopsis* and its relatives (Lysak *et al.*, 2006; Mandakova and Lysak, 2008). Such a cytogenetic approach should enable more deep and detailed studies of the

organization, phylogeny and evolution of *Brachypodium* genomes and give some insight into the mechanisms that determine significant variation in chromosome number even between closely related species. It cannot be ruled out that large chromosome-specific pools of *B. distachyon* BAC clones will also be useful to some extent in cytogenetic studies of other, more distant grass genomes.

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