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Laying the cytotaxonomic foundations of a new model grass, *Brachypodium distachyon* (L.) Beauv.

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Abstract

Brachypodium distachyon is a ubiquitous, temperate grass species which is being developed and exploited as an alternative model to rice, in order to gain access to important syntenic regions of the genomes of less tractable relatives such as wheat. As part of this initiative, this paper describes for the first time the cytotaxonomy of members of the polyploid series of this species, and challenges the assumption that the series evolved simply by chromosome doubling. *In situ* hybridization using genomic DNA probes and rDNA markers uncovers a hybrid origin of several of the polyploid ecotypes, and sheds light upon the complex evolution of this species and its close relatives.

Introduction

Brachypodium is a genus of temperate grasses with a wide distribution and relatively few species, two features indicating a very ancient origin (Tateoka 1968). The phylogenetic status of *Brachypodium* has been controversial over the years, but a recent consensus based upon cytological, anatomical and physiological parameters places *Brachypodium* into its own tribe Brachypodieae of the family Poaceae. In support of this taxonomic classification, recent molecular phylogenetic investigations have consistently placed the Brachypodieae into the sub-family Pooideae alongside the 'core' pooid clade, which includes the most important temperate cereals such as wheat, barley and oats (Catalan *et al.* 1997, Catalan and Olmstead 2000, Catalan *et al.* 1995, Hsiao *et al.* 1995, Shi *et al.* 1993). Furthermore, it appears that the genus *Brachypodium* is a distinct clade, which diverged soon after the division of the Pooideae from the Oryzeae, and is therefore more closely related to the temperate cereals and grasses than is rice (Draper *et al.* 2001).

A collection of over 50 accessions of *B. distachyon* has been assembled in Aberystwyth by Brachyomics, a University company established to promote the characterization and distribution of *Brachypodium* germplasm (Jenkins *et al.* 2003a). These accessions have chromosome numbers ranging from 10 to 30 (Robertson 1981), variation which is typical of species in this genus and which explains the intraspecific differences in DNA amounts (Draper *et al.* 2001) and the uncertainties with respect to ploidy levels (Bennett and Leitch 1995).

Recent microdensitometric and flow cytometric measurements of four of the 15 diploid accessions

of *B. distachyon* with $2n = 2x = 10$ revealed that this species had the smallest reported genome size of the Poaceae (172 Mbp), comparable to that of the model plant *Arabidopsis thaliana* (Draper *et al*. 2001). These estimates are consistent with previous observations that species of *Brachypodium* have the smallest 5S rDNA spacer of the grasses, and contain typically less than 15% highly repetitive DNA (Catalan *et al*. 1995). On the basis of its unusually compact genome, short annual life cycle, inbreeding habit and other desirable features, *B. distachyon* was selected for development and exploitation as a model species for the temperate cereals and grasses (Draper *et al*. 2001). The ultimate aim of this initiative is to use *B. distachyon* as a 'bridge' to gain access to important syntenic regions in less tractable relatives such as wheat. This approach necessitates a clear demonstration of colinearity between *B. distachyon* and its relatives, and is currently being investigated by the sequencing and 'landing' of BAC clones of *B. distachyon* to this group of cereals and grasses.

The other accessions have higher multiples of 10 chromosomes which prompted Robertson (1981) to suggest that this species had evolved a polyploid series based upon $2n = 2x = 10$, and that ecotypes that deviate from multiples of 10 are aneuploids. Without detailed cytological analysis, it is of course feasible that some of the polyploids may have arisen by hybridization and chromosome doubling. The distinction between autopolyploidy and allopolyploidy is important in the context of the evolution of this species, and influences the extent to which *B. distachyon* can be used as a model to investigate pairing and recombination in polyploids. For example, we know that one polyploid (ABR100) forms 15 bivalents at metaphase I of meiosis (Jenkins *et al*. 2003b). However, because the genomic constitution of this polyploid is unknown, we do not know if this is achieved by association of pairs of homologues in an autopolyploid, or by a restriction upon homoeologous pairing in an allopolyploid. So this work was undertaken in order to determine the likely genomic constitution of five putative polyploid ecotypes, using genomic *in situ* hybridization (GISH) and FISH with rDNA markers.

Material and methods

Plant material

Details about the ecotypes of *B. distachyon* and its diploid relative used in this study are given in Table 1.

Chromosome preparation

Seeds were germinated on filter paper moistened with tap water at 22.5°C in the dark for 2–3 days. To ensure optimal condensation of chromosomes at metaphase, whole seedlings with roots 1.0–2.0 cm long were immersed in ice-cold water for 24 hours, then fixed in 3 : 1 (v/v) methanol : glacial acetic acid and stored at − 20°C. Fixed seedlings were washed in 0.01 mol/L citric acid–sodium citrate buffer (pH 4.6–4.8) for 20 min prior to digestion in an enzyme mixture comprising 20% (v/v) pectinase

Table 1. The original identities, somatic chromosome numbers (2n), sources and origins of the ecotypes of *B. distachyon* and related diploid used in this study.

Accession No.	Species	2n	Source	Origin
ABR1	*B. distachyon*	10	Stace[1]	Kaman, Kiresihir, Turkey
ABR114	*B. distachyon*	20	Stace	Formenterra, Spain
ABR100	*B. distachyon*	30	Stace	Kalafabad, Iran
ABR112	*B. distachyon*	30	Stace	Corse, Leigem, Belgium
ABR113	*B. distachyon*	30	Stace	Lisbon, Portugal
ABR117	*B. distachyon*	30	USDA (219965)	Afghanistan
–	*B. sylvaticum*	20	Griffith/Gwynn-Jones[2]	Ireland

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(Sigma), 1% (w/v) cellulase (Calbiochem) and 1% (w/v) cellulase 'Onozuka R-10' (Serva) for 2 h at 37°C. Meristems were dissected out from root tips and squashed in drops of 45% acetic acid. Coverslips were removed by freezing and the preparations post-fixed in 3 : 1 ethanol : glacial acetic acid, followed by dehydration in absolute ethanol and air drying.

DNA probes

The following probes were used in this study:

(i) For GISH, total genomic DNA from ABR1 and ABR114 was extracted from young plants using a standard CTAB (cetyltrimethylammonium bromide) procedure. Non-sheared genomic DNA was labelled by nick translation with either tetramethyl-rhodamine-5-dUTP or digoxigenin-11-dUTP (Roche).

(ii) The 5S rDNA probe was generated from the wheat clone pTa794 (Gerlach and Dyer 1980) by PCR amplification and labelled also by PCR with tetramethyl-rhodamine-5-dUTP (Roche). The oligonucleotide primers and conditions for this reaction were as follows: universal M13 forward (5′-CAG GGT TTT CCC AGT CAC GA-3′) and reverse (5′-CGG ATA ACA ATT TCA CAC AGG A-3′) sequencing primers, 93°C × 5 min, 43 cycles of 94°C × 30 s, 55°C × 30 s, 72°C × 90 s, 1 cycle of 72°C × 5 min.

(iii) The 25S rDNA probe was made by nick translation of a 2.3 kb *Cla*I sub-clone of the 25S rDNA coding region of *A. thaliana* (Unfried and Gruendler 1990) with digoxigenin-11-dUTP (Roche).

Fluorescence in situ *hybridization*

The FISH procedure was adapted from Hasterok *et al.* (2002). The slides were pretreated with RNase (100 μg/ml) in 2 × SSC at 37°C for 1 h, washed in 2 × SSC and dehydrated in ethanol. The hybridization mixture consisted *inter alia* of 50% deionised formamide, 2 × SSC, salmon sperm blocking DNA in 75–100 × excess of labelled probe and 2.5–3.0 ng/μl of each DNA probe. No unlabelled genomic DNA was used as a block in any GISH experiment on the polyploid species. Reciprocal GISH experiments were performed using genomic DNA probes from each ecotype being compared, in order to validate pairwise comparisons between different ecotypes and to avoid misinterpretation of genomic relationships. Chromosome preparations and the denatured (75°C for 10 min) hybridization mixture were then denatured together at 70°C for 4.5 min in an Omnislide *in situ* hybridization system (Hybaid) and allowed to hybridize overnight in a humid chamber at 37°C. After hybridization, slides were washed stringently for 10 min in 15% deionized formamide in 0.1X SSC at 42°C, followed by immunodetection of digoxigenated probes by FITC-conjugated anti-digoxigenin antibodies (Roche). The chromosomes were mounted and counterstained in Vectashield (Vector Laboratories) containing 2.5 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

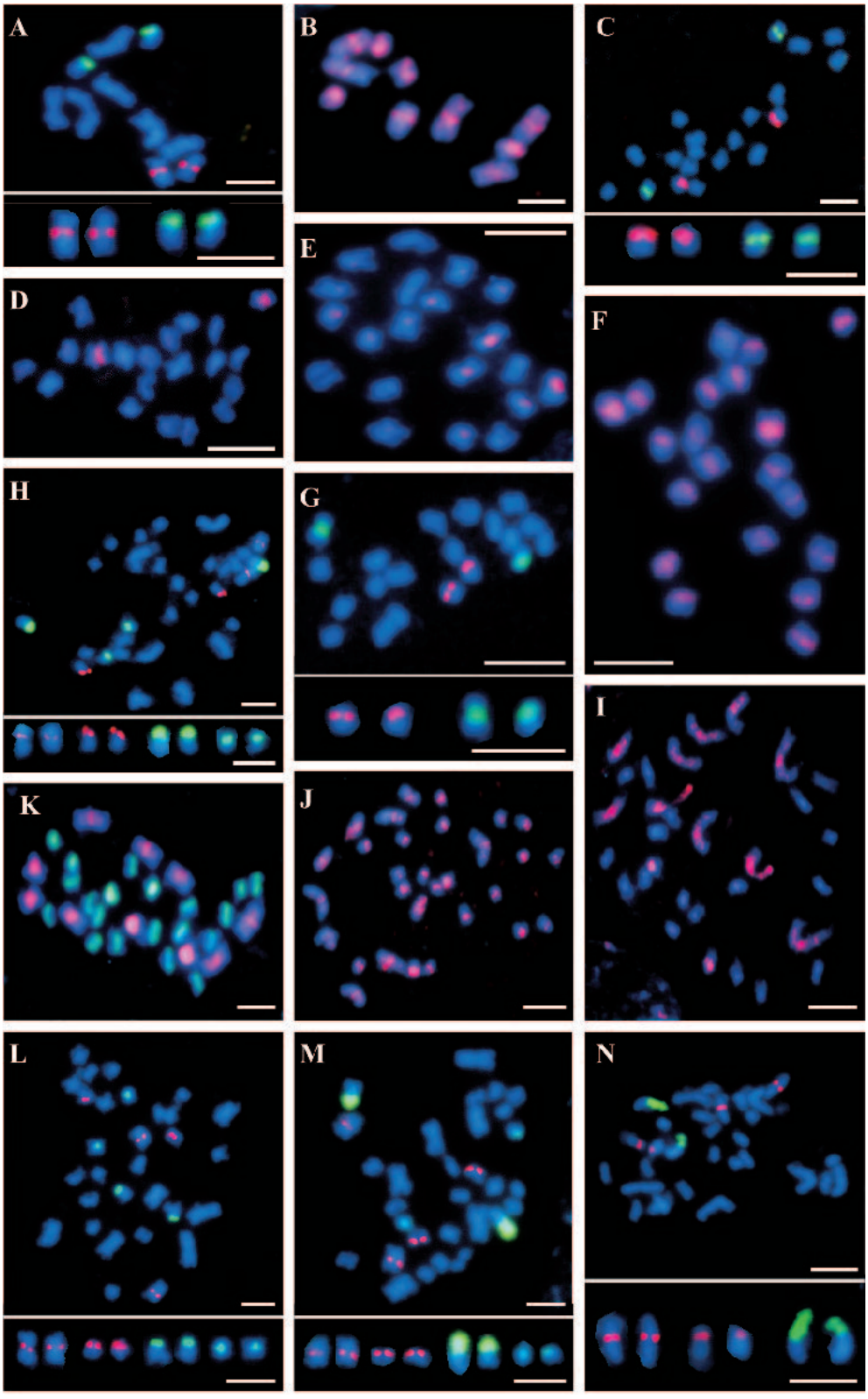
Image acquisition and processing

All images were taken with a Hamamatsu ORCA monochromatic CCD camera attached to a Zeiss Axioplan epifluorescence microscope, tinted using Wasabi software (Hamamatsu Photonics), and processed uniformly and superimposed using Micrografx (Corel) Picture Publisher software.

Results

The 10 chromosomes of ABR1 are shown in Figure 1A. One pair has a major 5S rDNA locus in the proximal region of its long arm, and another pair bears the only 45S locus at the distal region of its short arm (Figure 1A). *In situ* hybridization with genomic DNA of ABR1 to somatic chromosomes of the same species reveals signals that are predominantly, but not exclusively, concentrated in pericentromeric regions and at rDNA loci (Figure 1B).

ABR114 is a putative autotetraploid with 20 chromosomes. However, it has a more symmetrical karyotype and its chromosomes are considerably smaller than ABR1. Furthermore, it has the same number of 5S and 45S rDNA loci as ABR1, the latter being distinctly more proximal than those of ABR1 (Figure 1C). GISH with genomic DNA from ABR114 shows the expected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concentration of signals around the centromeres and at the four rDNA loci (not shown). GISH with genomic DNA of ABR1 to the chromosomes of ABR114 failed to highlight any region, except two putative rDNA loci (Figure 1D). The reciprocal experiment gave faint pericentromeric signals on six of the 10 chromosomes of ABR1, and two distal signals on chromosome 5 at the 45S rDNA loci (not shown). These results demonstrate that a diploid genotype like ABR1 is unlikely to be the progenitor of ABR114. A more likely possibility is that ABR114 is a closely related but different diploid with $2n = 2x = 20$.

To explore this possibility further, genomic DNA from both ABR1 and ABR114 were hybridized separately to the chromosomes of a related diploid species *B. sylvaticum*, our accession of which was known to be $2n = 2x = 20$. Genomic DNA of ABR1 faintly labelled some of the pericentromeric regions and two rDNA loci of the chromosomes of *B. sylvaticum* (Figure 1E). By contrast, genomic DNA of ABR114 labelled the chromosomes of *B. sylvaticum* (Figure 1F) with an intensity comparable to that to the chromosomes of ABR114 itself (not shown). In addition, the karyotype of ABR114 is clearly much more like *B. sylvaticum* in terms of the relative sizes of its chromosomes and the positions of its rDNA loci (Figure 1G). The conclusion is, therefore, that at this level of resolution ABR114 is more like *B. sylvaticum* than is ABR1.

ABR113 has 30 chromosomes and is considered to be an autohexaploid in the polyploid series of *B. distachyon*. Examination of its chromosome complement reveals that it does not have the morphological profile expected of a karyotype comprising three genomes of *B. distachyon*. For example, the smaller chromosomes are too numerous for this to be the case (Figure 1H). In addition, this genotype contains two pairs of chromosomes bearing 5S rDNA loci and two pairs with 45S, not six pairs in total expected on the basis of autohexaploidy. Also, the size and position of one 5S and one 45S locus are distinctly different compared to ABR1 (Figure 1H). GISH with the DNA and chromosomes of ABR113 reveals a pattern of signals expected on the basis of localization of repetitive DNA (not shown). Hybridization of genomic DNA from ABR1 to ABR113 clearly distinguished the 10 largest chromosomes of the complement, together with two smaller rDNA-bearing chromosomes of the other complement (Figure 1I). The results of the reciprocal GISH experiment are as expected (not shown), and the conclusion is that ABR113 contains a diploid genome similar in DNA composition to ABR1. GISH with genomic DNA from ABR114 to ABR113 chromosomes lights up the other remaining 20 smaller chromosomes (Figure 1J), and the results of the reciprocal experiment is as expected with all chromosomes showing signals (not shown). The conclusion is that ABR113 also contains a genome similar in composition to ABR114. In short, the results show that ABR113 contains two distinctly different genomes, one similar to ABR1 and the other similar to ABR114. The genomic composition of ABR113 is most clearly demonstrated when genomic DNA from both ABR1 and ABR114 are used simultaneously as probes to the chromosomes of ABR113 (Figure 1K). The 5S and 45S rDNA loci were also mapped on three other accessions of *B. distachyon* with 30 chromosomes. ABR112 has smaller distal 45S rDNA loci (Figure 1L) compared with ABR113 (Fig. 1H), ABR100 has a pair of greater and a pair of smaller (Figure 1M), and ABR117 has two fewer (Figure 1N).

Discussion

ABR1 has five pairs of chromosomes, each of which can be distinguished unambiguously on the basis of morphology and distribution of rDNA loci. Six other diploid ecotypes (ABR2-7)

Figure 1. Double target FISH of 5S rDNA (red fluorescence) and 25S rDNA (green fluorescence) probes to somatic metaphase chromosomes of (**A**) ABR1 ($2n = 10$), (**C**) ABR114 ($2n = 20$), (**G**) *B. sylvaticum* ($2n = 20$), (**H**) ABR113, (**L**) ABR112, (**M**) ABR100 and (**N**) ABR117 (all $2n = 30$). *In situ* hybridization of ABR1 genomic DNA (red) to somatic chromosomes of (**B**) ABR1, (**D**) ABR114, (**E**) *B. sylvaticum*, and (**I**) ABR113. ABR114 total genomic DNA (red) hybridized to somatic metaphase chromosomes of (**F**) *B. sylvaticum*, and (**J**) ABR113. (**K**) Double target GISH of ABR1 (red) and ABR114 (green) total genomic DNA probed to ABR113 chromosomes. The chromosomes are counterstained with DAPI (blue). All scale bars represent 5 μm.

share the same karyotype, which permitted the construction of a consensus karyotype for the diploid of this species (Draper *et al.* 2001). Apparent structural uniformity of these diploids enabled the confident selection of accessions ABR1 and ABR5 as the model genotypes, from which two BAC libraries have recently been prepared for functional genomic studies. The preponderance of repetitive DNA in the pericentromeric regions shown by GISH to somatic chromosomes of ABR1 with genomic DNA of the same species, reflects the compactness and economy of its genome, and reinforces its selection as a useful model species.

ABR114 was considered to be an autotetraploid form of *B. distachyon*, on the basis of its appearance and chromosome number of 20. However, the molecular cytogenetic study above shows that it is much more likely to be a related diploid with a genome similar in composition to *B. sylvaticum*. Whilst a classification error cannot be ruled out, the two species are quite distinct and can be separated on the basis of morphology and anatomy using conventional taxonomic criteria. It is, of course, possible that it is a hitherto unclassified sub-type of one of these species, although its somatic chromosome number of 20 is different from the 18 recorded for *B. sylvaticum* in other studies (Bennett and Smith 1991, Aragon-Alcaide *et al*. 1996, Draper *et al*. 2001).

The inference from this study is that ABR113 is not an autohexaploid, but rather is an allotetraploid resulting from the natural hybridization of two diploid progenitors resembling genomically ABR1 and ABR114. Artificial hybrids have been produced between *B. distachyon* and perennial European species of *Brachypodium* (such as *B. sylvaticum*), but it was noted that meiosis was very irregular resulting in infertility (Khan and Stace 1999). All four ecotypes with 30 chromosomes in this study are fertile, and the one ecotype examined cytologically (ABR100) has normal meiosis and forms bivalents perfectly (Jenkins *et al*. 2003b). Clearly, the progenitors of the allotetraploid in this study are either more compatible in hybrids, or the hybrids themselves have evolved pairing control mechanisms similar to those of wheat and other allopolyploids. The separation of the genomes within the allotetraploid is obviously efficient, since there is no indication from GISH for recombination between homoeologous chromosomes. There may, therefore, be some advantage in developing and using the natural polyploid hybrids of *Brachypodium* for the isolation and characterization of diploidizing genes.

ABR113 appears to be a simple amalgam of two putative diploid progenitors similar to ABR1 and ABR114, at the level of resolution afforded by the FISH and GISH experiments described above. Whilst comparable GISH experiments have yet to confirm the allotetraploid status of the three other ecotypes with 30 chromosomes, FISH has shown that there are noticeable differences in organization of rDNA compared with ABR113. This could indicate either that one or both of the progenitors of these polyploids had a different rDNA constitution and that independent hybridization events had occurred, or that there has been restructuring of the genomes of the polyploids following an initial hybridization event. Neither of these explanations are unlikely given that variability of rDNA has been noted and reported in other plant species, such as in the genus *Brassica* (Hasterok *et al*. 2001, Kulak *et al*. 2002, Maluszynska and Hasterok 2004). At present, we only have rDNA markers with which to gauge the extent of structural variation between polyploid ecotypes and their progenitors. There could, of course, be more extensive rearrangement that is invisible. However, we are currently physically mapping bacterial artificial chromosome clones of *B. distachon* to particular chromosome arms that will provide markers for gross structural variation of linkage groups.

Because so few of the accessions have been analysed, it would be premature to conclude that *B. distachyon* does not have a polyploid series based upon $2x = 10$, and that polyploids arise only through hybridization followed by chromosome doubling. However, the converse is also worth bearing in mind, that phenotypic similarity and possession of multiples of 10 chromosomes do not necessarily confirm the autopolyploid status of this species. Clearly, there is much scope for further investigation of these accessions of *B. distachyon*, together with broader cytogenetic coverage of related species that may be implicated in the complex evolution of this genus.

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References

Aragon-Alcaide L, Miller T, Schwarzacher T, Reader S, Moore G (1996) A cereal centromeric sequence. *Chromosoma* **10**: 261–268.

Bennett MD, Leitch IJ (1995) Nuclear DNA amounts in angiosperms. *Ann Bot* **76**: 113–176.

Bennett MD, Smith JB (1991) Nuclear DNA amounts in angiosperms. *Phil Trans R Soc Lond B* **334**: 309–345.

Catalan P, Olmstead RG (2000) Phylogenetic reconstruction of the genus *Brachypodium* Beauv. (Poaceae) from combined sequences of chloroplast gene and nuclear ITS. *Plant System Evol* **220**: 1–19.

Catalan P, Shi Y, Armstrong L, Draper J, Stace CA (1995) Molecular phylogeny of the grass genus *Brachypodium* P-Beauv based on RFLP and RAPD analysis. *Bot J Linn Soc* **117**: 263–280.

Catalan P, Kellogg EA, Olmstead RG (1997) Phylogeny of Poaceae subfamily Pooideae based on chloroplast *ndh*F gene sequences. *Mol Phylogenet Evol* **8**: 150–166.

Draper J, Mur LAJ, Jenkins G, Ghosh-Biswas GC, Bablak P *et al.* (2001) *Brachypodium distachyon*. A new model system for functional genomics in grasses. *Plant Physiol* **127**: 1539–1555.

Gerlach WL, Dyer TA (1980) Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. *Nucl Acids Res* **11**: 4851–4865.

Hasterok R, Jenkins G, Langdon T, Jones RN, Maluszynska J (2001) Ribosomal DNA is an effective marker of *Brassica* chromosomes. *Theor Appl Genet* **103**: 486–490.

Hasterok R, Langdon T, Taylor S, Jenkins G (2002) Combinatorial labelling of DNA probes enables multicolour fluorescence *in situ* hybridization in plants. *Folia Histochem Cytobiol* **40**: 319–323.

Hsiao C, Chatterton NJ, Asay KH, Jensen KB (1995) Phylogenetic relationships of the monogenomic species of the wheat tribe, Triticeae (Poaceae), inferred from nuclear rDNA (internal transcribed spacer) sequences. *Genome* **38**: 211–223.

Jenkins G, Hasterok R, Draper J (2003a) Building the molecular cytogenetic infrastructure of a new model grass. In: Zwierzykowski Z, Surma M, Kachlicki P, eds. *Application of Novel Cytogenetic and Molecular Techniques in Genetics and Breeding of the Grasses*. Poznan: Institute of Plant Genetics, Polish Academy of Sciences, pp 77–84.

Jenkins G, Mur LAJ, Bablak P, Hasterok R, Draper J (2003b) Prospects for functional genomics in a new model grass. In: Leister D, ed. *Functional Genomics in Plants*. Haworth Press (in press)

Khan MA, Stace CA (1999) Breeding relationships in the genus *Brachypodium* (Poaceae: Pooideae). *Nord J Bot* **19**: 257–269.

Kulak S, Hasterok R, Maluszynska J (2002) Karyotyping of *Brassica* amphidiploids using 5S and 25S rDNA as chromosome markers. *Hereditas* **136**: 144–150.

Maluszynska J, Hasterok R (2004) Discrimination of the parental genomes in *Brassica juncea* using GISH and FISH. *Cytogenet Genome Res* (in press).

Robertson IH (1981) Chromosome numbers in *Brachypodium* Beauv. (Gramineae). *Genetica* **56**: 55–60.

Shi Y, Draper J, Stace CA (1993) Ribosomal DNA variation and its phylogenetic implication in the genus *Brachypodium* (Poaceae). *Plant System Evol* **188**: 125–138.

Tateoka T (1968) Phytogeographical notes on the genus *Brachypodium* P. Beauv. (Gramineae). *Bol Soc Arg Bot* **12**: 44–56.

Unfried I, Gruendler P (1990) Nucleotide sequence of the 5.8S and 25S rRNA genes and the internal transcribed spacers from *Arabidopsis thaliana*. *Nucl Acids Res* **18**: 4011.