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Edited by
Z. Zwierzykowski, M. Surma and P. Kachlicki

Institute of Plant Genetics, Polish Academy of Sciences

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**Z. Zwierzykowski, M. Surma and P. Kachlicki**

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# Building the molecular cytogenetic infrastructure of a new model grass

G. JENKINS[1*], R. HASTEROK[2], J. DRAPER[1]

*[1]Institute of Biological Sciences, Edward Llwyd Building, University of Wales, Penglais, Aberystwyth, Ceredigion, SY23 3DA UK; [*]gmj@aber.ac.uk; [2]Department of Plant Anatomy and Cytology, University of Silesia, Jagiellońska 28, 40-032 Katowice, Poland*

**Abstract.** *Brachypodium distachyon* has been identified as a potential model for the temperate cereals and grasses. Its favourable phylogenetic position within the Pooideae, together with desirable biological features such as a small, compact genome, short life cycle, inbreeding habit, and amenability to culturing, transformation and regeneration, has prompted its exploitation for functional genomics in this group of plants. Its cytology is poorly understood at present, which hampers investigations into chromosome-specific function and behaviour, and precludes the integration of physical and genetic maps. This paper describes the laying of the molecular cytogenetic foundations for this species, in order to realise its potential for gene discovery and isolation in its cereal and grass relatives.

## Focus on *Brachypodium*

*Brachypodium* is a genus of temperate grasses comprising only about 12-15 recognised species. These range across the Mediterranean and neighbouring regions extending to the East, with representatives identified in South America and South Africa. The wide distribution of relatively few species was interpreted by TATEOKA (1968) as an indication of a very ancient genus. Although the phylogenetic status of *Brachypodium* has been rather controversial over the years, the recent consensus based upon cytological, anatomical and physiological parameters (SHARMA 1979) is that *Brachypodium* belongs to its own tribe of Brachypodieae of the family Poaceae. The phylogenetic status of this group has recently been corroborated by molecular analysis of nuclear rDNA sequence variation (SHI et al. 1993; HSIAO et al. 1995), nuclear DNA polymorphism (CATALAN et al. 1995) and sequence variation at the 3' end of the chloroplast *ndh*F gene (CATALAN et al. 1995; CATALAN, OLMSTEAD 2000). In short, these molecular phylogenetic investigations have consistently placed the Brachypodieae into the subfamily Pooideae alongside the "core" pooid clade which includes the most important temperate cereals such as wheat, barley and oats (KELLOGG

1998). Furthermore, it appears that the genus *Brachypodium* is a distinctive clade which diverged soon after the division of the Pooideae from the Oryzeae, and therefore is more closely related to the temperate cereals and grasses than is rice (DRAPER et al. 2001).

## Search for a model species

The phylogenetic proximity of *Brachypodium* to an ancestral temperate grass stock, prompted an investigation of this genus, with a view to the identification and development of a grass species as a useful model representative of important temperate cereals and grasses. The favourable phylogenetic position of *Brachypodium* also means that there is likely to be a high degree of conservation of gene order and repertoire within this group, with relatively little risk of breakdown of microsynteny in orthologous chromosome regions of near relatives. This could be exploited for gene discovery and isolation by positional cloning, and the development of bioinformatic tools essential to the success of functional genomics programmes. However, a potential model should also possess biological features that make it more amenable to scientific investigation compared with the other less tractable members of the group it represents, such as small diploid genome, rapid life cycle, small stature and undemanding growth requirements, tractable genetics and mapping populations, together with efficient mutagenesis and transformation procedures. These features enable the relatively facile construction of a functional genomics toolkit comprising large insert genomic libraries, extensive DNA sequence information, dense physical and genetic maps, and transcriptome, proteome and metabolome datasets.

Examination of the Kew Angiosperm C-value Database (http://www.kew.org/cval/homepage.html) showed that the *Brachypodium* genus is distinct from other genera in the Pooideae in that all of its species examined to date have small genome sizes comparable to rice. Indeed, this feature together with its favourable phylogenetic position, prompted the use of *B. sylvaticum* in the search for archetypal grass centromere sequences in wheat, maize, rice and *Brachypodium* (ARAGON-ALCAIDE et al. 1996). More recent microdensitometric measurements of two diploid accessions of *B. distachyon* revealed that this species had the smallest reported genome size in the Poaceae to date (DRAPER et al. 2001). Additional analysis by flow cytometry of four diploid accessions confirmed that this species had a 1C-value virtually indistinguishable from that of the model plant *Arabidopsis thaliana* (DRAPER et al. 2001). These estimates are consistent with previous observations that *Brachypodium* species have the smallest 5S rDNA spacer of the grasses, and contain typically less than 15% highly repeated DNA (CATALAN et al. 1995).

*B. distachyon* was selected as the model species within the genus on the basis of its exceptionally small genome size, short annual life cycle, inbreeding habit and other desirable features (DRAPER et al. 2001). A collection of over 50 accessions was assembled in Aberystwyth (Tab. 1) in order to test particular genotypes for vernalisation requirements, susceptibility to disease, ease of culture, transformation and regeneration, and other characters important in the development of a model biological system. Diploids were derived mainly from western Europe, only one tetraploid identified from the island of Formenterra (Spain), and hexaploids collected to the east of Europe. Two particular genotypes (ABR1 and ABR5) were selected as representatives of the diploids, and one genotype (ABR100) for the hexaploids.

## Getting to know its chromosomes

Another desirable attribute of a model organism is a karyotype in which the chromosomes are low in number and readily identifiable. These features enable the integration of physical and genetic maps, and open up avenues of research into chromosome-specific structure and function. The genus *Brachypodium* is unusual in that it contains species with basic chromosome numbers of 5, 7, 8 and 9 (ROBERTSON 1981), in contrast to the usual 7 in the majority of the Pooideae. *B. distachyon* has a basic chromosome number of 5, which is probably the archetypal number of the temperate cereals and grasses, given the phylogenetic divergence of this species from an ancestral stock of the Pooideae just prior to the radiation of modern core pooids.

The somatic metaphase chromosomes were examined from seven different populations of the diploid *B. distachyon* (ABR1 to ABR7, Table 1), which all have a genome size less than 175 Mbp. No apparent structural polymorphism was detected between the seven ecotypes, which permitted for the first time the construction of a consensus karyotype for this species (DRAPER et al. 2001). The karyotype is fortunately sufficiently asymmetric to enable the unambiguous identification of three of the five pairs of chromosomes by conventional aceto-carmine staining and bright-field light microscopy. Chromosome 1 is submetacentric, distinctly the largest of the complement, and unlikely to be confused with chromosome 2, which is more acrocentric and considerably smaller. Chromosome 3 is shorter than 2 and is the only metacentric of the complement. Chromosome 4 is very similar to chromosome 3, with which it could be confused. However, fluorescence *in situ* hybridisation (FISH) reveals that the former only has a single major 5S rDNA locus located proximally in its long arm (Fig. 1a). Chromosome 5 is acrocentric and by far the smallest of the complement. It also bears the only 45S rDNA locus, which occupies a considerable distal por-

**Table 1.** The current collection of *Brachypodium distachyon* germplasm in Aberystwyth. Further information on accessions from USDA can be obtained from http://www.ars-grin.gov/cgi-bin/npgs/html/site.pl?W6

| New code | Old code | Source | Origin | Ploidy |
|---|---|---|---|---|
| ABR1 | B200 | Stace | Kaman, Kiresehir, Turkey | 2x |
| ABR2 | B306 | Stace | Octon, Herault, France | 2x |
| ABR3 | B373 | Stace | Huesca, Aisa, Spain | 2x |
| ABR4 | B374 | Stace | Huesca, Aren, Spain | 2x |
| ABR5 | B375 | Stace | Huesca, Jaca, Spain | 2x |
| ABR6 | B376 | Stace | Navarra, Los Arcos, Spain | 2x |
| ABR7 | B377 | Stace | Valladolid, Otero, Spain | 2x |
| ABR8 | B382 | Stace | Siena, Italy | 2x |
| ABR9 | B384 | Stace | Ljubljana, Croatia | 2x |
| ABR10 | B393 | Stace | Montes Serantes, Vizcaya, Spain | 2x |
| ABR11 | 170218 | USDA | Turkey, Soma, Manisa | 2x |
| ABR12 | 185133 | USDA | Iraq | 2x |
| ABR13 | 185134 | USDA | Iraq | 2x |
| ABR14 | 245730 | USDA | Turkey | 2x |
| ABR15 | 254867 | USDA | ? | 2x |
| ABR99 | B266 | Stace | Kaschmar, Iran | 6x |
| ABR100 | B199 | Stace | Kalafabad | 6x |
| ABR101 | B189 | Stace | Nr. Darling, Cape Province, S. Africa | 6x |
| ABR102 | B194 | Stace | Jabal-us-Siraj, Kabul, Afghanistan | 6x |
| ABR103 | B195 | Stace | Nr. Shustar, Iran | 6x |
| ABR104 | B201 | Stace | Nr. Pabbi, Pakistan | 6x |
| ABR105 | B202 | Stace | Nr. Ongda, Morocco | 6x |
| ABR106 | B207 | Stace | Uruguay | 6x |
| ABR107 | B221 | Stace | Greece | 6x |
| ABR108 | B206 | Stace | Nr. Carmel, Israel | ? |
| ABR109 | B269 | Stace | Iraq | 6x |
| ABR110 | B380 | Stace | Aude, France | 6x |
| ABR111 | B381 | Stace | Paris, France | 6x |
| ABR112 | B383 | Stace | Corse, Leigem, Belgium | 6x |
| ABR113 | B385 | Stace | Lisbon, Portugal | 6x |
| ABR114 | B392 | Stace | Formenterra, Spain | 4x |
| ABR115 | 208216 | USDA | South Africa | 6x |
| ABR116 | 219961 | USDA | Afghanistan | 6x |
| ABR117 | 219965 | USDA | Afghanistan | 6x |
| ABR118 | 219968 | USDA | Afghanistan | 6x |
| ABR119 | 219971 | USDA | Afghanistan | 6x |
| ABR120 | 225067 | USDA | Afghanistan | 6x |
| ABR121 | 226452 | USDA | Iran | 6x |
| ABR122 | 226629 | USDA | Iran | 6x |
| ABR123 | 227011 | USDA | Iran | 6x |
| ABR124 | 233228 | USDA | Israel | 6x |
| ABR125 | 239713 | USDA | Iran | 6x |
| ABR126 | 239714 | USDA | Iran | 6x |

**Table 1.** cont.

| New code | Old code | Source | Origin | Ploidy |
|---|---|---|---|---|
| ABR127 | 239715 | USDA | Iran | 6x |
| ABR128 | 239716 | USDA | Iran | 6x |
| ABR129 | 250647 | USDA | Pakistan | 6x |
| ABR130 | 253334 | USDA | Morocco | 6x |
| ABR131 | 254868 | USDA | Iraq | 6x |
| ABR132 | 287783 | USDA | Spain | 6x |
| ABR133 | 317417 | USDA | Afghanistan | 6x |
| ABR134 | 321403 | USDA | Israel | 6x |
| ABR135 | 372187 | USDA | Uruguay | 6x |
| ABR136 | 422452 | USDA | Germany | 6x |
| ABR137 | 533015 | USDA | W. Australia | 6x |
| ABR138 | - | Jenkins | Quinta da Pacheca, Portugal | ? |
| ABR140 | B387 | Stace | ? | 6x |
| ABR141 | B388 | Stace | ? | 6x |
| ABR142 | B403 | Stace | Castillion, France | ? |
| ABR143 | B295 | Stace | ? | ? |

tion of its short arm (Fig. 1a). In summary, the use of only one diagnostic chromosomal landmark ensures the unequivocal identification of all 5 chromosomes of the complement. The rDNA probes are also effective at interphase (Fig. 1b), and have some utility in exploring the nuclear disposition of this chromosome domain at this stage. FISH also shows that the telomeres of the chromosomes comprise tandem repeats of the typical consensus sequence (TTTAGGG)n (JENKINS et al. 2003), which colocalises with the telomeric sequence HT100.3 derived from the model plant *Arabidopsis thaliana* (Fig. 1c). Another useful chromosomal marker is the pericentromeric repeat sequence CCS1, originally derived from the related species *B. sylvaticum* (ARAGON-ALCAIDE et al. 1996), which reliably delimits the chromosome arms for mapping purposes. This FISH probe can also be used in conjunction with HT100.3 in order to explore the relative special distribution of these domains and the orientation of chromosomes throughout the mitotic cell cycle (Fig. 1d) and meiosis. The pericentromeric repeat CCS1 is a motif within a Ty3-*gypsy* class retrotransposon which appears to have colonised the centromeres of a wide range of species within the Poaceae (LANGDON et al. 2000). FISH with conserved regions of the reverse transcriptase (Fig. 1e) and the integrase (Fig. 1f) genes of this element confirmed that the pericentromeric regions of *B. distachyon* share similar structures to other members of the Poaceae. FISH also confirms that this class of retroelement constitutes a sizeable proportion of the repetitive DNA component of this species, since it is a large part of the genomic probe signal with which it colocalises at the centromere (Fig. 1g).

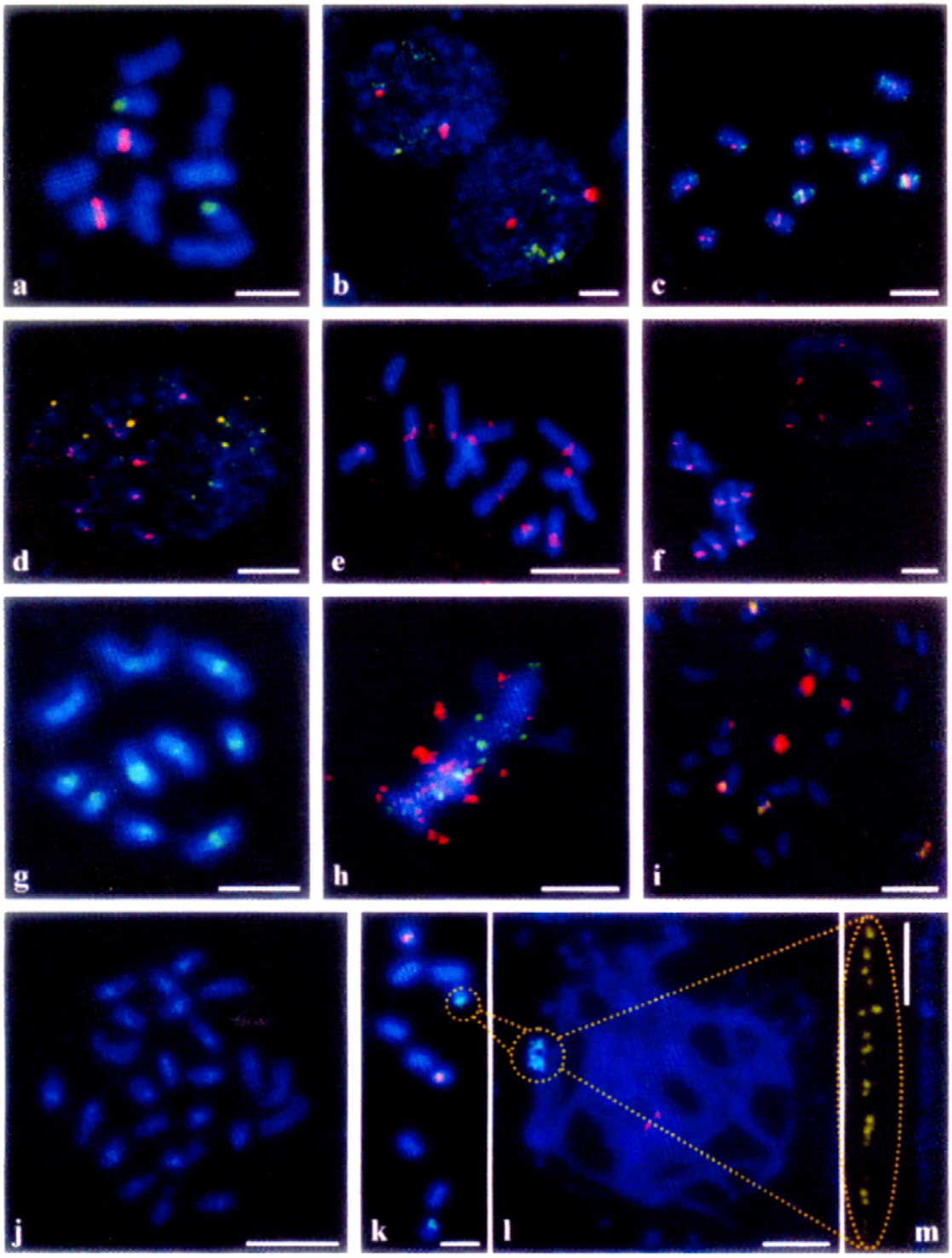


**Figure 1.** FISH images of ABR5 at somatic metaphase (a) and somatic interphase (b) showing 5S rDNA (red) and 25S rDNA (green) signals. FISH images of ABR1 at somatic metaphase (c) and somatic interphase (d) showing CCS1 pericentromeric (red) and HT100.3 telomeric (green) signals. Chromosome mapping in ABR1 of (e) the reverse transcriptase gene (red) and (f) the integrase gene (red) of the "crwydryn" retrotransposon element. (g) Genomic *in situ* hybridisation of ABR1 total genomic DNA (green) to ABR1 somatic metaphase chromosomes. (h) Probing of meiotic metaphase chromosomes of ABR1 with CCS1 (red) and HT100.3 (green) sequences. (i) FISH of rice BAC GLAb17124 (green), 25S rDNA (red) and 5S rDNA (yellow) onto mitotic chromosomes of rice (IR64). (j) FISH of rice BAC GLAy8686 (green) onto mitotic chromosomes of rice (IR64). (k-m) Enhanced resolution of the 25S rDNA signal (green) through the use of meiotic prophase chromosomes (l) and extended DNA fibres (m). Red signals on (k-l) visualise chromosomal localisation of 5S rDNA. All scale bars = 5 μm.

The immature inflorescence of *B. distachyon* is tiny compared with those at comparable stages in other members of the Poaceae and has correspondingly minute anthers. Nevertheless, there is a predictable gradation of development along the spike which permits the retrieval of particular meiotic stages and the construction of an atlas (JENKINS et al. 2003) comparable to that obtained in *Arabidopsis thaliana* (ROSS et al. 1996). The atlas confirms the regularity and orthodoxy of meiosis in this species, and will serve as a useful reference for the phenotypic description of meiotic mutants in this species as they become available. Examination of 20 pollen mother cells at metaphase I of the ABR1 ecotype revealed a chiasma frequency of about two per bivalent, which is surprisingly high for chromosomes of this size. This feature, together with apparently random distribution of chiasmata, are important in terms of the rapid generation of accurate, dense and comprehensive genetic and molecular marker maps. Meiotic chromosomes also respond favourably as substrates for FISH, as the cells at metaphase I (Fig. 1h) and prophase I (Fig. 1l) attest. The latter stage has particular utility in increasing the resolution of chromosome maps, since meiotic prophase chromosomes are considerably longer than their mitotic counterparts, and may reveal substructure in signals which is effectively invisible in mitotic chromosome preparations (Fig. 1k). Resolution may be further enhanced through the use of extended DNA fibres as FISH substrates, which have the potential at least to map the relative positions of single genes. Figure 1m shows extended DNA fibres of ABR1 probed with a 2.3 kbp fragment of the 10 kbp 45S rDNA subunit. The 10 kbp periodicity of the signal illustrates the potential for fine mapping of small contiguous DNA regions.

One of the ultimate aims of establishing *B. distachyon* as a new model, is to gain access to desirable genetic regions in this species by alignment with syntenic regions in the less tractable relatives. Colinearity may be inferred from the favourable phylogenetic positions of *B. distachyon* and the other temperate cereals and grasses, but such an approach necessitates a clear demonstration that the linear gene order is conserved at the microsyntenic level. Given the current dearth of sequence information and absence of a large insert library in this species, it was decided to test microsynteny in this species by the targeted landing of heterologous BACs onto the genome of this species. Microscale BAC isolation and DOP-PCR have been developed and used to successfully hybridise rice BACs to somatic chromosomes of the same species (Fig. 1i and j). However, FISH with 6 out of 24 rice BACs, each anchored to particular chromosome arms, have so far failed to map specifically and discretely to the chromosomes of *B. distachyon*. Such attempts may be confounded by the small but finite amount of repetitive DNA likely to be present in the large inserts of the rice BACs. Therefore, attention has now been turned to producing a BAC library in *B. distachyon* itself, which could be used not only for testing synteny in the grasses

and cereals, but also for future programs requiring the targeted sequencing of specific regions of the *B. distachyon* genome.

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