Research article

INTRASPECIFIC POLYMORPHISM OF RIBOSOMAL DNA LOCI NUMBER AND MORPHOLOGY IN *Brachypodium pinnatum* AND *Brachypodium sylvaticum*

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Abstract: The genus *Brachypodium* has become the target of extensive cytomolecular studies since one of its representatives, *B. distachyon*, has been accepted as a model plant for temperate cereals and forage grasses. Recent preliminary studies suggested that intraspecific rDNA polymorphism can occur in at least two members of the genus, *B. sylvaticum* and *B. pinnatum*, so the aim of this study was to further analyse this phenomenon. FISH with 25S rDNA and 5S rDNA probes was performed on somatic metaphase chromosomes, supplemented by the silver staining technique which distinguishes transcriptionally active from inactive 18S-5.8S-25S rDNA loci. The number, size and chromosomal distribution of 5S rDNA loci were very constant: two loci were invariably observed in all studied diploid accessions of both species, while four 5S rDNA loci were present in the tetraploid *B. pinnatum*. In contrast to 5S rDNA loci, those of the 35S rDNA were more variable. Two or three loci were observed in the diploid *B. pinnatum* and four in tetraploid accessions. In chromosome complements of *B. sylvaticum* accessions from two to six 35S rDNA sites were detected. Regardless of total rDNA locus number, only two were transcriptionally active in diploid accessions of both species, while two or four were active in the tetraploid *B. pinnatum*. Additionally, the fluorescent CMA/DAPI banding method was used to identify the relation between rDNA sites and CMA+ bands. It was revealed that the number and chromosomal distribution of CMA+ bands are in congruence only with 35S rDNA loci which gave strong FISH signals.

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INTRODUCTION

In almost every living cell ribosomal DNA (rDNA) sequences are actively transcribed, ensuring sufficient amounts of rRNA, which is an essential component of ribosomes. The genes encoding 18S, 5.8S and 25S rRNA (transcribed together as 35S preribosomal RNA) and those for 5S rRNA exist in a nuclear genome as arrays of tandem repeated units separated by non-transcribed spacers (NTSs), each of them at one or more loci in chromosomes [1]. In higher eukaryotes the 35S and 5S rDNA arrays may be found on separate chromosomes or may be closely linked on the same chromosome, whereas in some yeasts and liverworts these genes are physically interspersed within the same locus [2]. Every region coding for 18S, 5.8S, 25S rRNA and 5S rRNA is evolutionary conserved, but NTSs and ITSs (internal transcribed spacers, located on both sides of the 5.8S rDNA sequence) are more variable in terms of their length and sequence. This diversity within the noncoding sequences is highly informative in phylogenetic and taxonomic analysis [3-6]. rDNA sequences are also useful chromosome landmarks and provide important information about genome evolution at the chromosomal level. In some species, such as *Arabidopsis thaliana* [7], *Hordeum vulgare* [8] and *Rhoeo spathacea* [9, 10], fluorescence *in situ* hybridisation (FISH) with 25S and 5S rDNA as probes is sufficient to identify each chromosome in the complement. A complementary approach that can be used for karyotype characterization is double fluorescent banding with DAPI and CMA fluorochromes, which discriminates between AT- and GC-rich DNA regions. FISH experiments combined with CMA/DAPI staining facilitated effective identification of homologous chromosomes in uniform karyotypes of *Citrus paradisi* [11], *Lilium* [12] and *Pinus* [13].

rDNA sequences are widely used as chromosomal landmarks in cytogenetic studies. However, polymorphism of both rDNA loci number and position has been observed in many plant species, limiting the application of rDNA as reliable chromosome landmarks. Such intraspecific variation in number and chromosomal distribution of rDNA loci has been reported in numerous plant genera, including *Brassica*, *Raphanus*, *Sinapis* [14], *Pinus* [15] and *Sanguisorba* [16].

The number and chromosomal distribution of rDNA loci have also been analysed in some species of *Brachypodium* [17, 18]. This is a genus of temperate grasses, mostly Mediterranean and Eurosiberian, which comprises about 17 species [6, 19]. Molecular analysis [19-22] showed that *Brachypodium* is more closely related to key temperate cereals, such as wheat, rye, barley and oat, than rice. This feature, along with a very small nuclear genome (~355 Mb/1C DNA), low number (2n = 10) of easily distinguishable chromosomes and some attractive biological traits, such as undemanding growth requirements, self-fertility and short life cycle, have drawn attention to one of the representatives of

the genus, *B. distachyon*, which has become an internationally recognised model organism [23-25].

Brachypodium species possess variable base chromosome numbers of 5, 7, 8, 9 and 10 [6, 26] and rather small genomes [18]. Our previous cytogenetic studies in *Brachypodium* species showed that the number of both 5S and 35S rDNA loci varies from 2 to 6, while their position in chromosomes is highly conserved, 5S rDNA loci always being localised in pericentromeric regions while 35S rDNA are distributed at the chromosomal termini [18]. However, it turned out in this study that the number of rDNA loci may differ within a species, as was observed in *B. pinnatum* and *B. sylvaticum*. It was the first and so far the only evidence for the presence of polymorphism in the number of rDNA loci in those *Brachypodium* species. In this paper we extend this research to systematically analyse this phenomenon in several genotypes of both *B. pinnatum* and *B. sylvaticum*. Within the first species, both diploid and allotetraploid genotypes were analysed with one objective being to assess the changes in rDNA loci number that are possibly correlated with polyploidisation. Multicolour FISH with 5S rDNA and 25S rDNA on somatic metaphase chromosomes was performed to reveal all rDNA loci within the genome, with silver staining used to discriminate between active and inactive loci, and double staining with CMA and DAPI used to identify the spatial relationships between rDNA sites and CMA+ bands.

MATERIAL AND METHODS

Plant material
The *Brachypodium pinnatum* and *B. sylvaticum* accessions used in this study were obtained from the USDA-USA collection. Information about the origin of the seeds is listed in Table 1.

Table 1. Geographical origin of the investigated *Brachypodium* species.

Species	2n	Accession No.	Origin
Brachypodium pinnatum	18	PI 251803	Italy
		PI 380759	Iran
	28	PI 206677	Turkey
		PI 229676	Iran
Brachypodium sylvaticum	18	PI 23524	China
		PI 251102	Serbia and Montenegro
		PI 268222	Iran
		PI 269842	Tunisia
		PI 384810	Iran

Chromosome preparation
The somatic chromosome preparations were made as described by Hasterok *et al.*
[17]. Seeds were germinated in Petri dishes on moist filter paper at room
temperature (RT) in the dark. Seedlings with roots 1.5-2 cm long were immersed
in ice-cold water for 24 h, then transferred to a fixative (3:1 (v/v) methanol/glacial
acetic acid) and stored at -20°C until use. Excised roots were washed in 0.01M
citric acid-sodium citrate buffer (pH 4.8) and digested enzymatically at 37°C in
a mixture comprising 20% (v/v) pectinase (Sigma), 1% (w/v) cellulase
(Calbiochem) and 1% (w/v) cellulase 'Onozuka R-10' (Serva) for about two
hours. Multi-substrate chromosome preparations were prepared as described by
Hasterok *et al.* [27]. Briefly, three dissected meristems of each genotype were
transferred to a slide, in three separate drops (about 2 μl each) of 45% acetic
acid, then a 24 mm × 24 mm coverslip was gently applied to the slide and
squashed. Coverslips were removed by freezing and the preparations were post-
fixed in 3:1 ethanol/glacial acetic acid, dehydrated in absolute ethanol, air dried
and stored at -20°C.

CMA and DAPI staining
Double fluorescent staining with chromomycin A3 (CMA) and 4',6-diamidino-
2-phenylindole (DAPI) was performed as described by Schweizer [28]. Briefly,
preparations were stained with CMA solution (0.5 mg/ml, Sigma) at RT for an
hour in the dark, rinsed in distilled water and air dried. After subsequent staining
in DAPI solution (2.5 μg/ml, Sigma) at RT for 30 minutes, preparations were
briefly rinsed, air dried and mounted in a Dako antifade buffer.

Silver staining
To determine transcriptional activity of 18S-5.8S-25S rDNA sites the silver
staining technique according to Hizume *et al.* [29] was used. Preparations were
immersed in 0.01M borate buffer (pH 9.2; Merck) for 10 min at RT, and about
50 μl of freshly made 50% silver nitrate (Merck) in double distilled water was
applied to each slide. Then, slides covered with nylon meshes were incubated in
a humid chamber at 42°C for 20–25 min, washed in double distilled water, air
dried and mounted in glycerine. After imaging, slides for sequential silver
staining and FISH were rinsed in 4 × SSC at 37°C to remove coverslips and
mounting medium, washed in 30% hydrogen peroxide for 30 s to remove silver
staining, washed in distilled water and air dried.

DNA probes and FISH
Two ribosomal DNA sequences were used as probes: 5S rDNA (wheat clone
pTa794) [30], labelled by PCR with tetramethyl-rhodamine-5-dUTP (Roche),
and a 2.3 kb *Cla*I subclone of the 25S rDNA coding region of *Arabidopsis
thaliana* [31], labelled by nick-translation with digoxigenin-11-dUTP (Roche).
The 25S rDNA probe was used to detect 35S rRNA gene clusters. The details of
probe labelling and the following FISH procedure were as described by Hasterok
et al. [32] and Jenkins and Hasterok [33]. Slides were pre-treated with RNase

(100 µg/ml) in 2 × SSC at 37°C for 1 h, washed in 2 × SSC, dehydrated in ethanol and air dried. The hybridisation mixture consisted of 50% deionized formamide, 20% dextran sulphate, 2 × SSC, and sonicated salmon sperm blocking DNA in 50-100 × excess of labelled probes. Probes in a final concentration of about 2.5 ng/µl each were pre-denatured at 75°C for 10 min, then, after applying the mixture to slides, denatured together with chromosome material at 70°C for 4.5 min and allowed to hybridise in a humid chamber at 37°C overnight. After hybridisation slides were washed in 10% formamide in 2 × SSC (2 × 4 min, 42°C), which is equivalent to 79% stringency. Immunodetection of the digoxigenated probe was performed according to a protocol by Jenkins and Hasterok [33] using fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibodies (Roche). Slides were mounted in Vectashield (Vector Laboratories) containing 2.5 µg/ml DAPI.

Image acquisition and processing
All images were acquired using a monochromatic Photometrics CoolSNAP cf camera attached to a Leica DMRB epifluorescence microscope. Images were tinted using Wasabi (Hamamatsu Photonics) and processed uniformly and superimposed using Picture Publisher (Micrografx/Corel).

RESULTS AND DISCUSSION

The studies on the phenomenon of numerical variation of rDNA loci comprised four *B. pinnatum* and five *B. sylvaticum* accessions. Fifteen plants of each genotype/accession and at least ten metaphase plates from each individual were analysed. Because of the probability of polymorphism existing not only between accessions but also within a given accession, it was important to analyse each specimen individually. For this reason, multisubstrate chromosome preparations were performed [27], with three objects from different individuals on one slide separately, which ensured the same reaction conditions and made the analysis of number, as well as size and activity of rDNA sites more comparable and reliable.

Chromosome number and rRNA gene distribution
All studied accessions of *B. sylvaticum* had 18 chromosomes, while among *B. pinnatum* two different somatic chromosome numbers were observed, *i.e.* two accessions with 2n = 18 and another two with 2n = 28 (Table 2). Although Robertson [26] also described different chromosome numbers characteristic for those two species, *i.e.* 14, 28, 42, 44, and 56 for *B. sylvaticum* as well as 14 or 16 for *B. pinnatum*, the numbers observed in this study are in agreement with the results of Wolny and Hasterok [18]. Two different chromosome numbers found among *B. pinnatum* accessions suggest that polyploidisation may have happened during the formation of *B. pinnatum* with 28 chromosomes. The results of GISH analysis indicate species similar to present *B. pinnatum* (2n = 18) and *B. distachyon* (2n = 10) as putative phylogenetic ancestors of this hybrid [18]. However, recent molecular studies contradict this hypothesis and suggest that

both ancestral species had the chromosome number of 18 and some major rearrangements, *e.g.* dysploidy and/or partial diploidisation, took place after the formation of the new polyploid, leading to the present chromosome number of 28 [34].

Table 2. Number of chromosomes, CMA+ bands, total number of 5S and 35S rDNA sites and number of Ag-NORs in genomes of investigated *Brachypodium* species.

Accession No.	2n	5S rDNA	35S rDNA	Ag-NOR	CMA+ bands
B. pinnatum					
PI 251803	18	2	2	2	2
PI 380759	18	2	2 – 3	2	2
PI 206677	28	4	4	4	4
PI 229676	28	4	4	2	2
B. sylvaticum					
PI 23524	18	2	2	2	2
PI 251102	18	2	4	2	4
PI 268222	18	2	4 – 6	2	2
PI 269842	18	2	2 – 3	2	2
PI 384810	18	2	4	2	4

The exact distribution of rRNA gene sites was analysed using FISH with 5S and 25S rDNA as probes. Their simultaneous use revealed one pair of chromosomes carrying the 35S rRNA gene site and one pair of 5S rDNA in *B. pinnatum* accessions with 18 chromosomes, *i.e.* PI 380759 (Fig. 1A; Table 2) and PI 251803 (data not shown). The number of both types of rDNA sites was generally constant, but in two out of fifteen individuals of PI 380759 an additional 35S rDNA site was observed on a different chromosome of the complement (Fig. 1C). In interphase nuclei of those individuals, the additional hybridisation signal was substantially weaker, much more condensed than the other two and also localised outside the nucleoli (Fig. 1D). The chromosomal distribution of 35S rDNA sites in *B. pinnatum* 2n = 18 was always terminal, in contrast to 5S rDNA loci, which were situated in proximal regions of chromosomes. In nine individuals of the PI 251803 accession (data not shown) and six of PI 380759 (Fig. 1B) the chromosomal distribution of 18S-5.8S-25S rDNA loci was as expected, but an apparent difference in size and intensity of 25S rDNA signals was observed. The FISH signal in one chromosome of a homologous pair was much smaller and weaker than in the complementary one.

The two analysed genotypes of *B. pinnatum* 2n = 28 (PI 206677 and PI 229676) contained two chromosome pairs bearing terminally located 18S-5.8S-25S rDNA loci and two other pairs with pericentromeric 5S rDNA sites in their karyotypes (Fig. 1E - 1G; Table 2). Using morphometric differences it was possible to distinguish each chromosome pair carrying rDNA loci. No

significant variation in size or intensity of the 5S rDNA probe signal was observed; however, some differences were found in the fluorescence strength of the 25S rDNA probe. All studied individuals of the *B. pinnatum* PI 229676 genotype had two minor signals of 25S rDNA probe in the pair of longer chromosomes carrying these genes (Fig. 1E). Additionally, 13 out of 15 plants of the PI 206677 accession displayed 25S rDNA signals of similar size and intensity (Fig. 1F), whilst the last two individuals presented notably smaller and weaker signals in one homologue of the pair of longer chromosomes (Fig. 1G).

All studied *B. sylvaticum* accessions invariably had one pair of chromosomes with a pericentromeric distribution of 5S rDNA loci. In contrast, the number of 35S rDNA sites varied substantially (Table 2). Only one pair of chromosomes with these sequences was present in PI 23524 (Fig. 1H) as well as in the majority of analysed individuals of PI 269842 (Fig. 1I). Three sites of 35S rRNA genes were observed in one individual of PI 269842 (Fig. 1J), whilst four loci were seen in three accessions: PI 251102 (Fig. 1K), PI 384810 (Fig. 1L) and in some individuals of PI 268222 (Fig. 1O). This last genotype also included individuals with five (Fig. 1P) and six (Fig. 1Q) 35S rDNA sites (four and three individuals, respectively). Additionally, some differences in signal size and intensity as well as in chromosome position were revealed. All analysed individuals of PI 251102 had 25S rDNA signals of similar size, but in one chromosome pair, rDNA loci were located subterminally instead of at the expected terminal position (Fig. 1K; green arrows). In PI 384810, all 35S rDNA sites were located terminally, but three different types of signal size were distinguished. Firstly, eight individuals had two major signals in a pair of shorter chromosomes, while the minor loci were observed in two longer chromosomes (Fig. 1L). Secondly, in five specimens, three 35S rDNA sites of similar size and one significantly smaller rDNA locus were found. The minor locus was located in one of the longer chromosomes (Fig. 1M). Finally, two individuals with three minor and one major 25S rDNA signals were observed. The major signal was located in one of the shorter chromosomes bearing 35S rDNA sites, whilst its homologue and another pair of chromosomes contained minor loci (Fig. 1N). In the PI 268222 accession, which is polymorphic for rDNA number, two bigger and two smaller terminally located hybridisation signals of this probe were found in individuals with four 35S rDNA sites (Fig. 1O). In the complements of individuals with one or two chromosomes carrying additional 35S rDNA loci, the supernumerary rDNA sites were of intermediate size and located subterminally in the chromosomes; the total number of 35S rDNA loci was 5 (Fig. 1P) and 6 (Fig. 1Q), respectively.

To the best of our knowledge, the presence of two or three 35S rDNA loci in *B. sylvaticum* accessions has not been reported yet. However, this study along with the previous analysis [18] indicates that the most common number of 35S rRNA gene sites in this species is four. The results presented here confirmed the
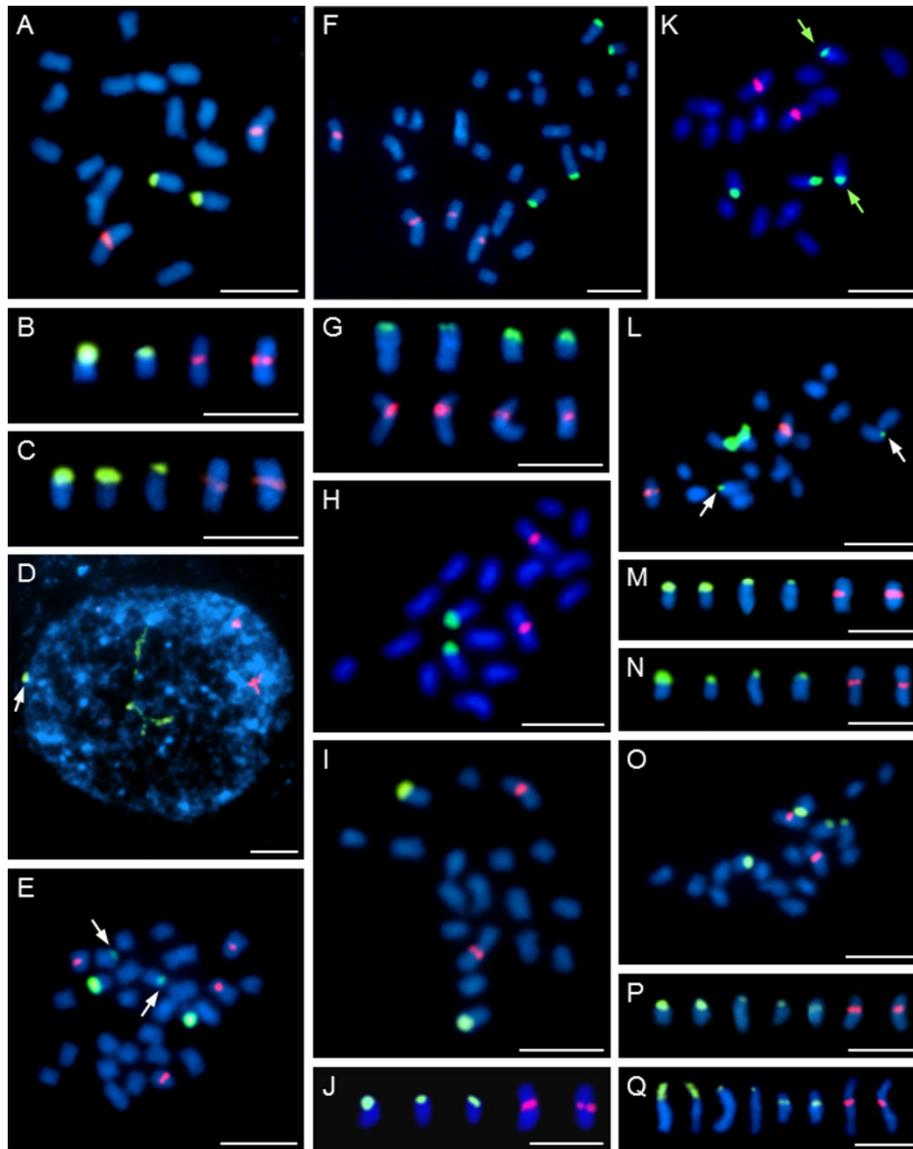
Fig. 1. Distribution of 5S (red) and 25S (green) ribosomal DNA sites determined by FISH to somatic metaphase chromosomes and interphase nucleus of *Brachypodium pinnatum* and *B. sylvaticum* accessions. A-D – *B. pinnatum* (2n = 18) PI 380759; E – *B. pinnatum* (2n = 28) PI 229676; F-G – *B. pinnatum* (2n = 28) PI 206677; H – *B. sylvaticum* PI 23524; I-J – *B. sylvaticum* PI 269842; K – *B. sylvaticum* PI 251102; L-N – *B. sylvaticum* PI 384810; O-Q – *B. sylvaticum* PI 268222. DAPI counterstaining – blue fluorescence. Arrows colour coding: white – minor terminal 35S rDNA loci; green – subterminal 35S rDNA loci. Scale bars = 5 μm.

occurrence of intraspecific variation of 35S rDNA loci number in both analysed species. Moreover, variability was also observed between individuals of a single accession. In *B. pinnatum* the number of 35S rDNA loci is much less variable, while the number of 5S rDNA sites in both analysed species is constant (Table 2). In general, FISH patterns of 35S rDNA are reported to be more polymorphic than those of 5S rDNA. Variation in chromosome patterns of 35S rRNA gene sites has been observed in many different plant species such as *Oryza sativa* subsp. *japonica* [35], *Centaurea jacea* [36] and *Phaseolus* species [37]. Variable loci of 5S rDNA have been reported, inter alia, in *A. thaliana* [7] and *Brassica juncea* var. *crispa* '685' [14]. 5S rDNA polymorphism was also observed in *Paphiopedilum* species, where massive duplication and amplification of 5S rDNA loci leading to large-scale polymorphism of number, size and physical position of signals was found [38]. On the other hand, strong conservation of rDNA locus number has been described in many plant species or even whole genera, *e.g. Glycine* [39] and *Setaria* [40].

rRNA genes are not found to be syntenic [41] and their copy number can change rapidly, which in some cases can result in complete elimination of entire loci or loss of most copies of 35S rDNA repeats, resulting in reduction or absence of a hybridisation signal. Various mechanisms have been proposed to explain the variability of rDNA sequences, *e.g.* chromosome rearrangements, gene conversion, unequal crossing-over or transposition by TE (transposable elements) [42, 43].

Apart from a polymorphism of 35S rDNA loci number in *Brachypodium* species, some variation in the intensity of 25S rDNA probe hybridisation signals was also observed. FISH is not a quantitative method, but it is acknowledged that variation in signal strength can to some extent reflect a different copy number of rDNA in particular loci [44]. No attempt was made to quantify FISH signal intensity in this study, but differences in signal intensity and size suggest an asymmetric distribution of 35S rDNA repeat number in *Brachypodium*. It was observed both between homologous and non-homologous 35S rDNA loci (Fig. 1B, 1G, 1M, 1N). In some cases one locus remains unbalanced (Fig. 1C, 1J, 1P). Differentiation of rDNA hybridisation signal size and intensity between chromosome pairs carrying these sequences is quite common in plants. It was observed *e.g.* in *Trifolium* [45], *Lupinus angustifolius* [46] and *Fragaria* species [47]. Variety in rDNA hybridisation signal intensity between homologues is not common, but it was reported in *Hypochaeris spathulata*, where the mechanism of the changes was postulated to be mainly unequal crossing-over [48].

It is also noticeable that in all examined accessions of *B. pinnatum* and *B. sylvaticum* chromosomal distributions of both kinds of rDNA sequences were conserved, while locus number and signal intensity of 25S rDNA were variable. 35S rRNA gene clusters were localised in terminal parts of chromosomes (or exceptionally in subterminal parts), while chromosomal distribution of 5S rDNA sites was always pericentromeric. Such localisation in chromosomes can also be connected with the frequency of polymorphism. It seems reasonable that changes in terminal parts of chromosomes affect chromosome pairing during

meiosis less than such events that occur close to the centromeres. Moreover, the distal position of rDNA may facilitate sequence association during meiosis that could lead to unequal exchanges of rDNA repeats and eventually a change of copy number at both homologous and non-homologous sites. Variation in locus number limited only to terminally located rDNA sequences was observed in *Phaseolus* [49] and *Fragaria* [47], but in some species, *e.g. Brassica*, both types of rDNA are highly variable, irrespective of their chromosomal position [14].

One of the objectives of this work was to assess whether changes in rDNA loci number are correlated with polyploidisation in *B. pinnatum* (2n = 28). All analysed genotypes of *B. pinnatum* (2n = 28) revealed only 4 loci of both types of rDNA sequence, indicating no rDNA loci number reduction or repeat amplification, with one exception, where diminution of 35S rDNA copy number repeats was visible as a smaller FISH signal in one polyploid accession of *B. pinnatum* (PI 229676). One pair of both kinds of rDNA loci in diploid chromosome complements of *Brachypodium* seems to be the ancestral number. Additional 35S rDNA loci which we observed in *B. sylvaticum* and *B. pinnatum* (2n = 18) genotypes probably arose during the evolution of this genus. These results indicate that 35S rDNA sequences are subjected to various changes in copy number and organisation in *Brachypodium* chromosomes.

Activity of rRNA genes

The silver staining technique is commonly used to discriminate transcriptionally active 18S-5.8S-25S rRNA genes from inactive ones and helps to complement results achievable by fluorescence *in situ* hybridisation, which identifies all 18S-5.8S-25S rDNA sites in a karyotype regardless of expression status. In this study silver staining was performed to visualise active rDNA loci in the investigated accessions of *B. pinnatum* and *B. sylvaticum* (Table 2). In both species, Ag-NORs were present only in terminal segments of chromosomes. Karyotypes of *B. pinnatum* (2n = 18) contained two active rDNA sites. Silver staining revealed two positive Ag bands that were of equal size or significantly asymmetric in size (Fig. 2A and Fig. 2B, respectively), consistent with the equivalent FISH pattern and further supporting diversity in the number and/or activity of 35S rDNA repeats. Similarly, one of the *B. pinnatum* (2n = 28) accessions (PI 229676) had a single pair of chromosomes bearing active rDNA sites, although possessing four rDNA loci in total (Fig. 2C). However, one pair of chromosomes in this accession displayed a very weak FISH signal of the 25S rDNA probe (Fig. 1E), so it is also possible that the amount of transcription-related proteins which are detected by staining with silver was below the detection level of this method. In the other genotype with 28 chromosomes (PI 206677) four chromosomes with positive Ag-NOR bands were observed (Fig. 2D). Four active rDNA sites in *B. pinnatum* (2n = 28) karyotypes were also observed by Wolny [50]. The other species analysed, *B. sylvaticum*, invariably had two Ag-NOR bands, even if more than two 35S rDNA sites were present
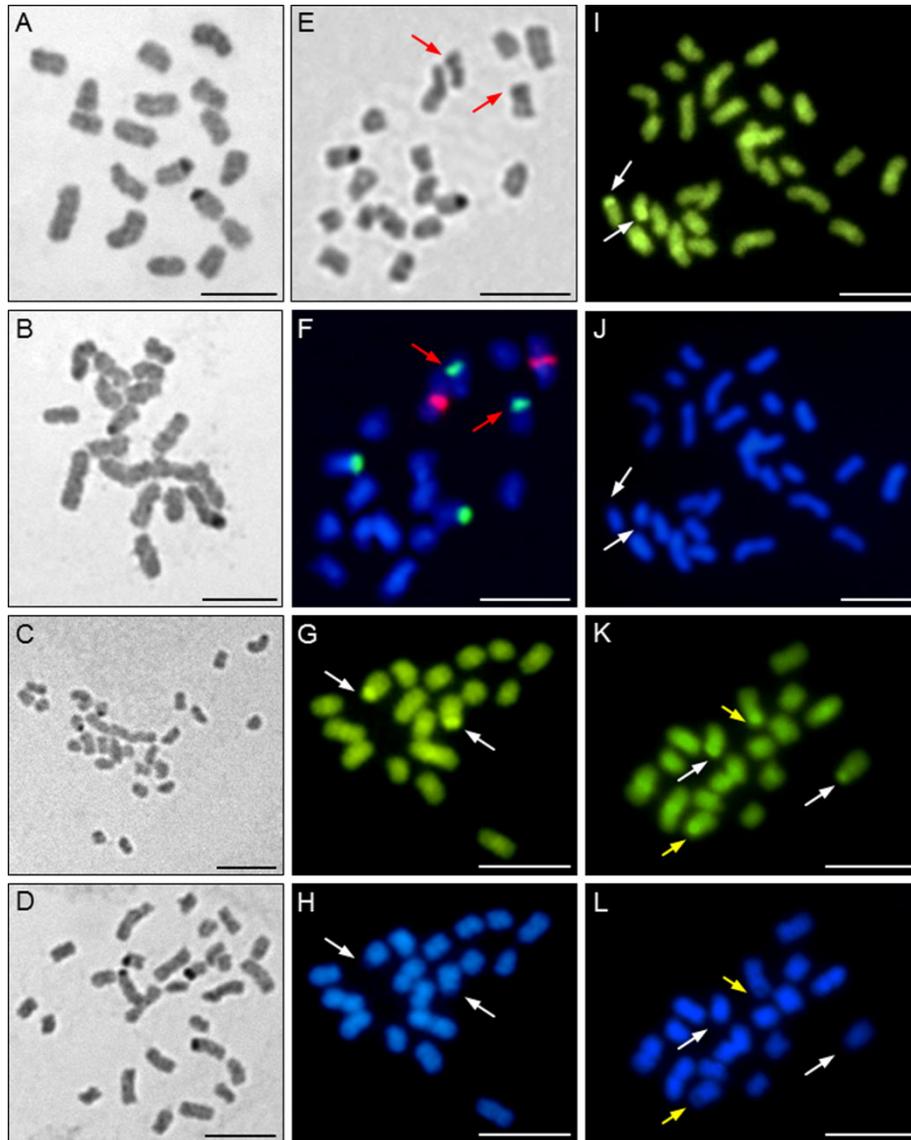
Fig. 2. Chromosomes of *Brachypodium pinnatum* and *B. sylvaticum* analysed by silver staining, FISH and CMA/DAPI techniques. A-D – silver staining of: A-B – *B. pinnatum* (2n = 18) PI 380759; C – *B. pinnatum* (2n = 28) PI 229676; D – *B. pinnatum* (2n = 28) PI 206677; E-F – sequential silver staining and FISH with 5S (red) and 25S (green) probes to metaphase chromosomes of *B. sylvaticum* PI 251102; G-H – Double fluorescent CMA/DAPI staining of *B. pinnatum* (2n = 18) PI 251803; I-J – CMA/DAPI of *B. pinnatum* (2n = 28) PI 229676; K-L – CMA/DAPI of *B. sylvaticum* PI 251102. CMA – yellow/green fluorescence; DAPI counterstaining – blue fluorescence. Arrows colour coding: white – terminal CMA+/DAPI- bands; yellow – subterminal CMA+/DAPI- bands; red – inactive 35S rDNA sites. Scale bars = 5 μm.

within a genome. Sequential silver staining and FISH, which allow identification of chromosomes containing active and inactive rDNA loci in the same chromosome complement, revealed that in the accessions of *B. sylvaticum* with more than two 35S rRNA gene sites only the ones localised in the pair of shorter chromosomes were transcriptionally active (Fig. 2E - 2F). The presence of inactive rDNA sites was reported for many other plant species, *e.g. Lupinus cosentinii* [46], three diploid *Brassica* species of the U-triangle [51] and allotetraploid *Brachypodium hybridum* (ABR113) [52].

Distribution of CMA+ bands

Double fluorescent staining with DAPI/CMA is used to localise chromosome regions rich in AT and GC base pairs, respectively [28]. In both examined species no DAPI+ bands were found, but DAPI- bands that corresponded to CMA+ regions were observed (Fig. 2G - 2L). In *B. pinnatum* 2n = 18 (Fig. 2G - 2H), one of the *B. pinnatum* accessions with 28 chromosomes (PI 229676; Fig. 2I - 2J) and three *B. sylvaticum* genotypes (PI 23524, PI 268222 and PI 269842; Table 2) only two bright CMA+ bands of equal size were present, irrespectively of the number of rDNA sites. Two pairs of chromosomes with CMA+ bands were seen in *B. pinnatum* 2n = 28 (PI 206677; data not shown) and two *B. sylvaticum* accessions (PI 251102 and PI 384810), which was equal to the number of 35S rDNA loci in those genotypes. Consistent with the silver staining results, all visualised CMA+ bands were localised terminally. The only exception was one pair of chromosomes in the PI 251102 accession of *B. sylvaticum*, which possessed two subterminal CMA+ bands (Fig. 2K - 2L; yellow arrows). The correlation between CMA+ bands and 35S rDNA sequences in plants is well known and was reported for species such as *Helianthus annuus* [53]. In species that form the 'U triangle' within the *Brassica* genus, the number of CMA+ bands was shown to correspond to the number of 35S rDNA loci, irrespectively of their size [54]. However, DAPI/CMA staining is much less sensitive than FISH and it is probable that this, combined with the small size of *Brachypodium* chromosomes, accounts for the lower number of CMA+ bands compared to the total number of 35S rRNA gene loci observed in some accessions here (Table 2), with some terminally located loci being too small for successful detection by DAPI/CMA staining. Consequently, this kind of chromosome staining in *Brachypodium* may only be appropriate for major rDNA sites. In other species, *e.g. Allium victorialis*, similar results were seen, as CMA+ regions were correlated only with the biggest pair of 35S rDNA loci [55].

REFERENCES

1. Weider, L.J., Elser, J.J., Crease, T.J., Mateos, M., Cotner, J.B. and Markow, T.A. The functional significance of ribosomal (r)DNA variation: impacts on the evolutionary ecology of organisms. **Annu. Rev. Ecol. Evol. Syst.** 36 (2005) 171-184.

2. Sone, T., Fujisawa, M., Takenaka, M., Nakagawa, S., Yamaoka, S., Sakaida, M., Nishiyama, R., Yamato, K.T., Ohmido, N., Fukui, K., Fukuzawa, H. and Ohyama, K. Bryophyte 5S rDNA was inserted into 45S rDNA repeat units after the divergence from higher land plants. **Plant. Mol. Biol.** 41 (1999) 679-685.

3. Adams, S.P., Leitch, I.J., Bennett, M.D., Chase, M.W. and Leitch, A.R. Ribosomal DNA evolution and phylogeny in *Aloe* (Asphodelaceae). **Am. J. Bot.** 87 (2000) 1578-1583.

4. Dhar, M.K., Friebe, B., Kaul, S. and Gill, B.S. Characterization and physical mapping of ribosomal RNA gene families in *Plantago*. **Ann. Bot.** 97 (2006) 541-548.

5. Dubouzet, J.G. and Shinoda, K. Phylogenetic analysis of the internal transcribed spacer region of Japanese *Lilium* species. **Theor. Appl. Genet.** 98 (1999) 954-960.

6. Catalan, P., Muller, J., Hasterok, R., Jenkins, G., Mur, L.A., Langdon, T., Betekhtin, A., Siwinska, D., Pimentel, M. and Lopez-Alvarez, D. Evolution and taxonomic split of the model grass *Brachypodium distachyon*. **Ann. Bot.** 109 (2012) 385-405.

7. Fransz, P., Armstrong, S., Alonso-Blanco, C., Fischer, T.C., Torres-Ruiz, R.A. and Jones, G. Cytogenetics for the model system *Arabidopsis thaliana*. **Plant J.** 13 (1998) 867-876.

8. Taketa, S., Harrison, G.E. and Heslop-Harrison, J.S. Comparative physical mapping of the 5S and 18S-25S rDNA in nine wild *Hordeum* species and cytotypes. **Theor. Appl. Genet.** 98 (1999) 1-9.

9. Golczyk, H., Hasterok, R. and Joachimiak, A.J. FISH-aimed karyotyping and characterization of Renner complexes in permanent heterozygote *Rhoeo spathacea*. **Genome** 48 (2005) 145-153.

10. Golczyk, H., Hasterok, R. and Szklarczyk, M. Ribosomal DNA, tri- and bi-partite pericentromeres in the permanent translocation heterozygote *Rhoeo spathacea*. **Cell. Mol. Biol. Lett.** 15 (2010) 651-664.

11. de Moraes, A.P., dos Santos Soares Filho, W. and Guerra, M. Karyotype diversity and the origin of grapefruit. **Chromosome Res.** 15 (2007) 115-121.

12. Siljak-Yakovlev, S., Peccenini, S., Muratovic, E., Zoldos, V., Robin, O. and Valles, J. Chromosomal differentiation and genome size in three European mountain *Lilium* species. **Plant Syst. Evol.** 236 (2003) 165-173.

13. Hizume, M., Shibata, F., Matsusaki, Y. and Garajova, Z. Chromosome identification and comparative karyotypic analyses of four *Pinus* species. **Theor. Appl. Genet.** 105 (2002) 491-497.

14. Hasterok, R., Wolny, E., Hosiawa, M., Kowalczyk, M., Kulak-Ksiazczyk, S., Ksiazczyk, T., Heneen, W.K. and Maluszynska, J. Comparative analysis of rDNA distribution in chromosomes of various species of Brassicaceae. **Ann. Bot.** <u>97</u> (2006) 205-216.

15. Liu, Z.-L., Zhang, D., Hong, D.-Y. and Wang, X.-R. Chromosomal localization of 5S and 18S-5.8S-25S ribosomal DNA sites in five Asian pines using fluorescence *in situ* hybridization. **Theor. Appl. Genet.** <u>106</u> (2003) 198-204.

16. Mishima, M., Ohmido, N., Fukui, K. and Yahara, T. Trends in site-number change of rDNA loci during polyploid evolution in *Sanguisorba* (Rosaceae). **Chromosoma** <u>110</u> (2002) 550-558.

17. Hasterok, R., Jenkins, G. and Draper, J. Laying the cytotaxonomic foundations of a new model grass, *Brachypodium distachyon* (L.) Beauv. **Chromosome Res.** <u>12</u> (2004) 397-403.

18. Wolny, E. and Hasterok, R. Comparative cytogenetic analysis of the genomes of the model grass *Brachypodium distachyon* and its close relatives. **Ann. Bot.** <u>104</u> (2009) 873-881.

19. Catalan, P. and Olmstead, R.G. Phylogenetc reconstruction of the genus *Brachypodium* P. Beauv. (Poaceae) from combined sequences of chloroplast *ndh*F gene and nuclear ITS. **Plant Syst. Evol.** <u>220</u> (2000) 1-19.

20. Shi, Y., Draper, J. and Stace, C. Ribosomal DNA variation and its phylogenetic implication in the genus *Brachypodium* (Poaceae). **Plant Syst. Evol.** <u>188</u> (1993) 125-138.

21. Catalan, P., Shi, Y., Armstrong, L., Draper, J. and Stace, C.A. Molecular phylogeny of the grass genus *Brachypodium* P. Beauv. based on RFLP and RAPD analysis. **Bot. J. Linn. Soc.** <u>117</u> (1995) 263-280.

22. Catalan, P., Kellogg, E.A. and Olmstead, R.G. Phylogeny of Poaceae subfamily Pooideae based on chloroplast *ndh*F gene sequences. **Mol. Phylogenet. Evol.** <u>8</u> (1997) 150-166.

23. Draper, J., Mur, L.A., Jenkins, G., Ghosh-Biswas, G.C., Bablak, P., Hasterok, R. and Routledge, A.P. *Brachypodium distachyon*. A new model system for functional genomics in grasses. **Plant Physiol.** <u>127</u> (2001) 1539-1555.

24. Garvin, D.F., Gu, Y.Q., Hasterok, R., Hazen, S.P., Jenkins, G., Mockler, T.C., Mur, L.A. and Vogel, J.P. Development of genetic and genomic research resources for *Brachypodium distachyon*, a new model system for grass crop research. **Crop Sci. - The Plant Genome** <u>48</u> (2008) 69-84.

25. International Brachypodium Initiative. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. **Nature** <u>463</u> (2010) 763-768.

26. Robertson, I.H. Chromosome numbers in *Brachypodium* Beauv. (Gramineae). **Genetica** <u>56</u> (1981) 55-60.

27. Hasterok, R., Dulawa, J., Jenkins, G., Leggett, M. and Langdon, T. Multi-substrate chromosome preparations for high throughput comparative FISH. **BMC Biotechnol.** <u>6</u> (2006) 20.

28. Schweizer, D. Reverse fluorescent chromosome banding with chromomycin and DAPI. **Chromosoma** 58 (1976) 307-324.
29. Hizume, M., Sato, S. and Tanaka, A. A highly reproducible method of nucleolus organizing regions staining in plants. **Stain Technol.** 55 (1980) 87-90.
30. Gerlach, W.L. and Dyer, T.A. Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. **Nucleic Acids Res.** 8 (1980) 4851-4865.
31. Unfried, I. and Gruendler, P. Nucleotide sequence of the 5.8S and 25S rRNA genes and of the internal transcribed spacers from *Arabidopsis thaliana*. **Nucleic Acids Res.** 18 (1990) 4011.
32. Hasterok, R., Langdon, T., Taylor, S. and Jenkins, G. Combinatorial labelling of DNA probes enables multicolour fluorescence *in situ* hybridisation in plants. **Folia Histochem. Cytobiol.** 40 (2002) 319-323.
33. Jenkins, G. and Hasterok, R. BAC 'landing' on chromosomes of *Brachypodium distachyon* for comparative genome alignment. **Nat. Protoc.** 2 (2007) 88-98.
34. Wolny, E., Lesniewska, K., Hasterok, R. and Langdon, T. Compact genomes and complex evolution in the genus *Brachypodium*. **Chromosoma** 120 (2010) 199-212.
35. Chung, M.C., Lee, Y.I., Cheng, Y.Y., Chou, Y.J. and Lu, C.F. Chromosomal polymorphism of ribosomal genes in the genus *Oryza*. **Theor. Appl. Genet.** 116 (2008) 745-753.
36. Dydak, M., Kolano, B., Nowak, T., Siwinska, D. and Maluszynska, J. Cytogenetic studies of three European species of *Centaurea* L. (Asteraceae). **Hereditas** 146 (2009) 152-161.
37. Pedrosa, A., Vallejos, C., Bachmair, A. and Schweizer, D. Integration of common bean (*Phaseolus vulgaris* L.) linkage and chromosomal maps. **Theor. Appl. Genet.** 106 (2003) 205-212.
38. Lan, T. and Albert, V.A. Dynamic distribution patterns of ribosomal DNA and chromosomal evolution in *Paphiopedilum*, a lady's slipper orchid. **BMC Plant Biol.** 11 (2011) 126.
39. Singh, R.J., Kim, H.H. and Hymowitz, T. Distribution of rDNA loci in the genus *Glycine* Willd. **Theor. Appl. Genet.** 103 (2001) 212-218.
40. Benabdelmouna, A., Abirached-Darmency, M. and Darmency, H. Phylogenetic and genomic relationships in *Setaria italica* and its close relatives based on the molecular diversity and chromosomal organization of 5S and 18S-5.8S-25S rDNA genes. **Theor. Appl. Genet.** 103 (2001) 668-677.
41. Shishido, R., Sano, Y. and Fukui, K. Ribosomal DNAs: an exception to the conservation of gene order in rice genomes. **Mol. Gen. Genet.** 263 (2000) 586-591.
42. Lim, K.Y., Kovarik, A., Matyasek, R., Bezdek, M., Lichtenstein, C.P. and Leitch, A.R. Gene conversion of ribosomal DNA in *Nicotiana tabacum* is associated with undermethylated, decondensed and probably active gene units. **Chromosoma** 109 (2000) 161-172.

43. Raskina, O., Belyayev, A. and Nevo, E. Quantum speciation in *Aegilops*: molecular cytogenetic evidence from rDNA cluster variability in natural populations. **Proc. Natl. Acad. Sci. U.S.A.** 101 (2004) 14818-14823.
44. Leitch, I.J. and Heslop-Harrison, J.S. Physical mapping of the 18S-5.8S-26S rRNA genes in barley by *in situ* hybridization. **Genome** 35 (1992) 1013-1018.
45. Ansari, H.A., Ellison, N.W., Reader, S.M., Badaeva, E.D., Friebe, B., Miller, T.E. and Williams, W.M. Molecular cytogenetic organization of 5S and 18S-26S rDNA loci in white clover (*Trifolium repens* L.) and related species. **Ann. Bot.** 83 (1999) 199-206.
46. Hajdera, I., Siwinska, D., Hasterok, R. and Maluszynska, J. Molecular cytogenetic analysis of genome structure in *Lupinus angustifolius* and *Lupinus cosentinii*. **Theor. Appl. Genet.** 107 (2003) 988-996.
47. Liu, B. and Davis, T.M. Conservation and loss of ribosomal RNA gene sites in diploid and polyploid *Fragaria* (Rosaceae). **BMC Plant Biol.** 11 (2011) 157.
48. Weiss-Schneeweiss, H., Stuessy, T., Siljak-Yakovlev, S., Baeza, C.M. and Parker, J. Karyotype evolution in South American species of *Hypochaeris* (Asteraceae, Letuceae). **Plant Syst. Evol.** 241 (2003) 171-184.
49. Moscone, E.A., Klein, F., Lambrou, M., Fuchs, J. and Schweizer, D. Quantitative karyotyping and dual-color FISH mapping of 5S and 18S-25S rDNA probes in the cultivated *Phaseolus* species (Leguminosae). **Genome** 42 (1999) 1224-1233.
50. Wolny, E. [Cytogenetyczna analiza porównawcza wybranych gatunków *Brachypodium*]. PhD thesis, University of Silesia, Katowice, Poland. (2008).
51. Hasterok, R. and Maluszynska, J. Cytogenetic analysis of diploid *Brassica* species. **Acta Biol. Cracov. Ser. Bot.** 42 (2000) 145-153.
52. Idziak, D. and Hasterok, R. Cytogenetic evidence of nucleolar dominance in allotetraploid species of *Brachypodium*. **Genome** 51 (2008) 387-391.
53. Cuellar, T., Belhassen, E., Fernandez-Calvin, B., Orellana, J. and Bella, J.L. Chromosomal differentiation in *Helianthus annuus* var. *macrocarpus*: heterochromatin characterization and rDNA location. **Heredity** 76 (1996) 586-591.
54. Hasterok, R. and Maluszynska, J. Nucleolar dominance does not occur in root tip cells of allotetraploid *Brassica* species. **Genome** 43 (2000) 574-579.
55. Seo, B.B., Lee, S.H. and Mukai, Y. Physical mapping of 5S and 18S-26S ribosomal RNA gene families in *Allium victorialis* var. *platyphyllum*. **J. Plant Biol.** 40 (1997) 132-137.