

Diversity of a Complex Centromeric Satellite and Molecular Characterization of Dispersed Sequence Families in Sugar Beet (*Beta vulgaris*)

GERHARD MENZEL^{1,†}, DARYNA DECHYEVA^{1,†}, TORSTEN WENKE¹,
DANIELA HOLTGRÄWE², BERND WEISSHAAR² and THOMAS SCHMIDT^{1,*}

¹Institute of Botany, Dresden University of Technology, D-01062 Dresden, Germany and ²Institute of Genome Research, University of Bielefeld, D-33594 Bielefeld, Germany

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- **Background and Aims** The aim of this work was the identification and molecular characterization of novel sugar beet (*Beta vulgaris*) repetitive sequences to unravel the impact of repetitive DNA on size and evolution of *Beta* genomes via amplification and diversification.
- **Methods** Genomic DNA and a pool of *B. vulgaris* repetitive sequences were separately used as probes for a screening of high-density filters from a *B. vulgaris* plasmid library. Novel repetitive motifs were identified by sequencing and further used as probes for Southern analyses in the genus *Beta*. Chromosomal localization of the repeats was analysed by fluorescent *in situ* hybridization on chromosomes of *B. vulgaris* and two other species of the section *Beta*.
- **Key Results** Two dispersed repetitive families pDvul1 and pDvul2 and the tandemly arranged repeat family pRv1 were isolated from a sugar beet plasmid library. The dispersed repetitive families pDvul1 and pDvul2 were identified in all four sections of the genus *Beta*. The members of the pDvul1 and pDvul2 family are scattered over all *B. vulgaris* chromosomes, although amplified to a different extent. The pRv1 satellite repeat is exclusively present in species of the section *Beta*. The centromeric satellite pBV1 by structural variations of the monomer and interspersions of pRv1 units forms complex satellite structures, which are amplified in different degrees on the centromeres of 12 chromosomes of the three species of the *Beta* section.
- **Conclusions** The complexity of the pBV1 satellite family observed in the section *Beta* of the genus *Beta* and, in particular, the strong amplification of the pBV1/pRv1 satellite in the domesticated *B. vulgaris* indicates the dynamics of centromeric satellite evolution during species radiation within the genus. The dispersed repeat families pDvul1 and pDvul2 might represent derivatives of transposable elements.

Key words: *Beta vulgaris*, dispersed repeats, satellite DNA, FISH.

INTRODUCTION

Repetitive sequence families are major components of plant genomes (Heslop-Harrison, 2000). They are divided into tandemly arranged and dispersed sequences according to their genome organization (Schmidt and Heslop-Harrison, 1998). Tandem repeats include satellite DNA, micro- and minisatellites, telomeric repeats and ribosomal genes. Typical satellite repeating units are 160–180 bp or 320–360 bp long and are arranged in tandem arrays of hundreds of thousands copies (Hemleben *et al.*, 2000; Macas *et al.*, 2002), thus comprising a significant portion of the repetitive DNA. Satellites, together with transposable elements, are a major driving force for the variation in the plant genome regarding size and complexity (Heslop-Harrison, 1996, 2000). Dispersed elements are scattered over the genome and interspersed with other genomic sequences. Many of these repeats are derived from mobile DNA sequences, in particular from retrotransposons, as a result of an erroneous reverse transcription or divergence and rearrangements of integrated elements at the DNA level (Bennetzen *et al.*, 1994; SanMiguel *et al.*, 1996).

Repetitive DNA is predominantly localized in heterochromatic and subtelomeric regions of chromosomes (Heslop-Harrison, 1996). Particularly, plant centromeres, which are detectable as primary constrictions or as AT- or GC-rich heterochromatin, consist of various classes of repetitive sequences often separated by islands of higher complexity (Presting *et al.*, 1998; Copenhaver *et al.*, 1999; Heslop-Harrison *et al.*, 1999; Gindullis *et al.*, 2001a). Centromere-associated repeats have been described in various monocots (Dong *et al.*, 1998; Nagaki *et al.*, 1998; Hudakova *et al.*, 2001) and dicots (Harrison and Heslop-Harrison, 1995; Schmidt and Heslop-Harrison, 1996; Brandes *et al.*, 1997; Gindullis *et al.* 2001b).

The fast evolution of both tandemly arranged and dispersed repetitive DNA leads to changes in sequence and abundance (Schmidt and Heslop-Harrison, 1998) and often results in species-specific repeat variants and/or the generation of novel sequence families. Therefore, comparative studies of plant repetitive sequences are useful for the investigation of evolutionary relationships between plant species (Kamm *et al.*, 1995; Bennetzen, 2000; Ohmido *et al.*, 2000; Nouzova *et al.*, 2001).

Species of the genus *Beta* are grouped into four sections *Beta*, *Corollinae*, *Nanae* and *Procumbentes*. All cultivated beets (sugar, fodder, garden and leaf beet) belong exclusively

* For correspondence. E-mail thomas.schmidt@tu-dresden.de

† These authors contributed equally to this work.

to the section *Beta*. With approx. 20 closely and distantly related species and subspecies, the genus provides a suitable system for the comparative study of nuclear genome composition and evolution. Many genus-, section- or species-specific repetitive DNA sequences have been analysed from cultivated and wild *Beta* species (Schmidt and Metzloff, 1991; Kubis *et al.*, 1997, 1998; Gao *et al.*, 2000).

The sugar beet (*Beta vulgaris*) genome is 758 Mbp in size (Arumuganathan and Earle, 1991) and is estimated to contain 63 % repetitive sequences (Flavell *et al.*, 1974). In this paper, the genomic organization of novel dispersed and tandemly arranged repetitive sequences are described. In particular, analysis of BAC-end sequences revealed structural modifications of a centromere-specific satellite including the formation of a complex centromeric tandem array of unusual monomere size. The chromosomal organization of these repeat families was analysed by multicolour fluorescent *in situ* hybridization (FISH), and their distribution within the genus *Beta* was elucidated by comparative Southern hybridization.

MATERIALS AND METHODS

Plant material and DNA preparation

Plants of *Beta vulgaris* ssp. *vulgaris* 'KWS 2320' (sugar beet), 'Brigadier' (fodder beet), 'Rote Kugel' (garden beet) and 'Lukullus' (leaf beet), *Beta vulgaris* ssp. *maritima*, *Beta vulgaris* ssp. *adanensis*, as well as the species *Beta patula*, *Beta macrocarpa*, *Beta corolliflora*, *Beta macrorhiza*, *Beta nana*, *Beta procumbens*, *Beta patellaris* and *Spinacia oleracea*, were grown under greenhouse conditions. Genomic DNA was isolated from young leaves using the CTAB (cetyltrimethylammonium bromide) standard protocol (Saghai-Marooof *et al.*, 1984).

Isolation of repetitive DNA

For the isolation of repetitive DNA families, the plasmid library SGZR00841 from genomic *Beta vulgaris* DNA (genotype 'KWS 2320'), consisting of 27 648 clones with an average insert size of 0.5 kb spotted in duplicate onto nylon membranes (Menzel *et al.*, 2006), was subsequently probed with radiolabelled genomic DNA of *B. vulgaris* 'KWS2320' and a pool of all known radiolabelled repetitive *B. vulgaris* sequences. The overnight hybridizations were performed at 60 °C in 5× SSPE with 5× Denhardt solution and 0.2 % SDS. Post-hybridization washings were performed twice at 60 °C in 1× SSC/0.1 % SDS for 10 min. The signals of both hybridization experiments were analysed with the software VisualGrid 3.4.1.1000 (www.gpc-biotech.com).

Plasmid DNA of randomly chosen clones not hybridizing to the pool of known *B. vulgaris* repeats was further purified with the GFX Micro Plasmid Prep Kit (GE Healthcare, Chalfont St Giles, UK), and sequenced with a CEQ 8000 capillary sequencer (Beckman Coulter, Fullerton, CA, USA) according to the manufacturers instructions. Sequences were aligned by the MegAlign option of the Lasergene 6.0 software (DNASTar, Madison, Wisconsin) using CLUSTAL with default parameters.

PCR conditions

To generate probes for Southern hybridization, PCR reactions with 50 ng template DNA and a final primer concentration of 0.5 μM were performed in a 50-μL volume containing 0.2 mM dNTPs, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0) and 1 unit of Taq DNA polymerase (GE Healthcare). Standard PCR conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 49 °C to 55 °C for 45 s, 72 °C for 3 min 30 s and a final incubation at 72 °C for 10 min. For the amplification of a pDvu11 probe, the primers 5'-ACCCGTATTTTCGTCTTTCTT-3' and 5'-TCGCCCCGTGTGATAATG-3' were used for PCR with the clone 44K4 as template at 50 °C annealing temperature. A probe representing the pDvu2 repeat family was amplified from the plasmid DNA of clone 17H23 with the primers 5'-GGAAACTTTTCCCATACCA-3' and 5'-GGGTTTTTT AGTTGAAATCT-3' at 50 °C annealing. Hybridizations with pRv1 were performed with a probe amplified from the plasmid clone 46C21 with the primer pair 5'-CGGATTT TATTGATAGAATG-3' and 5'-CTTAGATAAGACTATT CATGC-3' at an annealing temperature of 50 °C. A pBV1 probe was amplified from a cloned monomer sequence (EMBL accession Z22849) with the primer pair 5'-TTCAA TGCAACGCACTCG-3' and 5'-ATCCTCTAGTTCTCGAG C-3' using an annealing temperature of 54 °C.

After gel electrophoresis, PCR fragments were purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany).

Southern hybridization

For Southern hybridization, 10 μg genomic DNA was restricted with different enzymes, separated on 1.2 % agarose gels, and transferred onto Hybond-N⁺ nylon membranes (GE Healthcare) using alkaline transfer. Southern hybridizations using ³²P-labelled probes were performed using standard protocols (Sambrook *et al.*, 1989). Filters were hybridized at 60 °C and washed at 60 °C in 2× SSC/0.1 % SDS and 1× SSC/0.1 % SDS for 10 min each. Signals were detected by autoradiography.

FISH

The meristem of young leaves from *B. vulgaris* plants was used for the preparation of mitotic chromosomes. The material was macerated in an enzyme mixture and dropped onto slides as described by Schwarzacher and Heslop-Harrison (2000) with modifications (Desel, 2002). The probes were labelled with biotin-11-dUTP or digoxigenin-16-dUTP by PCR (Schwarzacher and Heslop-Harrison, 2000), and hybridization and detection were performed according to Schmidt *et al.* (1994). Signal amplification was performed with a Thyramide Amplification Kit according to manufacturer's instructions (Molecular Probes, Invitrogen). Chromosome preparations were counterstained with DAPI (4',6'-diamidino-2-phenylindole) and mounted in antifade solution (CitiFluor).

Examination of slides was carried out with a Zeiss Axioplan2 fluorescence microscope equipped with filters

09 (FITC), 15 (Cy3) and 01 (DAPI). Images were acquired directly with the Applied Spectral Imaging v. 3.3 software coupled with the high-resolution CCD camera ASI BV300-20A. The images were contrast optimized using only functions affecting the whole image equally and printed using Adobe Photoshop 7.0 software.

RESULTS

Identification and sequence analysis of novel dispersed DNA families

To identify novel *B. vulgaris* repetitive DNA families, high-density filters of the plasmid library SGZR00841 containing approx. 15 Mbp were subsequently hybridized with genomic *B. vulgaris* DNA and most previously characterized *B. vulgaris* repeat families (Table 1). Comparing the number of hybridizing clones in each experiment, this differential hybridization approach revealed that 84 % of repetitive DNA sequences within the *B. vulgaris* genome had been characterized previously. Plasmid clones hybridizing strongly to genomic DNA, but not cross-reacting with known repeats were sequenced. Those with no significant similarities to other sequences in the EMBL database represented novel repeats and could be grouped into three families designated pDvul1, pDvul2 and pRv1.

For the pDvul1 repetitive family, five clones (EMBL accessions AM177315 and AM228861–AM228864) ranging in size from 226 bp to 656 bp and sharing similarity between 74.2 % and 76.3 % were identified. Among those, the repeat pDvul1-2 represents a full-length repetitive unit of 656 bp.

The six members of the pDvul2 family (AM177316, AM228865 and AM944727–AM944730) are 187–291 bp

long and 75.5–94.4 % similar. The repeats pDvul2-5 and pDvul2-6 form a subgroup within the family by the presence of four positionally conserved insertions with an average length of 53 bp, 22 bp, 9 bp and 7 bp. The interspersion of pDvul1 and pDvul2 family members was observed on the plasmid clone 17H23, where the repeats pDvul1-1 and pDvul2-1 are spaced by a genomic region of 40 bp.

Structural variability and complexity of a centromeric satellite repeat

From the pRv1 repetitive family, 12 members (AM944555–AM944566) were identified. The repeats pRv1-1 to pRv1-12 have a similar length of 211–244 bp. The sequence similarity within the members of the pRv1 family ranges from 63.5 % to 93.2 %.

Sequence analyses of plasmid clones revealed the interspersion of pRv1 units with monomers of the satellite repeat pBV1. The tandem repeat pBV1 was first described by Schmidt and Metzloff (1991) as a centromeric *Bam*HI satellite with a monomer length of 327 bp (Fig. 1A). To unravel this organization of pBV1 and pRv1 units, the *B. vulgaris* entries in the EMBL database (McGrath *et al.*, 2004) were screened using a pBV1 query, and subsequently compared. First, remarkable structural changes have been identified within pBV1 monomers, which can be assigned to three variants. Beside tandemly arranged pBV1 units of 327 bp (Fig. 1A), pBV1 monomers were observed containing an internal duplication of a 60-bp sequence motif, resulting in an increased repeat unit of 380 bp (Fig. 1B). Moreover, 380-bp pRv1 units were interspersed with monomers of approx. 440 bp, which are formed by a triple amplification of the 60-bp sequence motif (Fig. 1B). In addition to these observed variations in the pBV1 monomer structure, homology searches with a pRv1 query sequence to 5720 end sequences of a BAC library from the *B. vulgaris* cultivar ‘KWS 2320’ (Hohmann *et al.*, 2003; D. Holtgräwe and B. Weisshaar, unpubl. res.) identified pBV1 units of 340 bp, each containing pRv1 monomers with an average length of 250 bp at conserved integration sites, thus suggesting the formation of a complex satellite unit of approx. 590 bp (Fig. 1C).

Genomic organization of repetitive families in B. vulgaris

Genomic organization of the repetitive DNA in *B. vulgaris* was analysed by Southern hybridization using representative members of the pDvul1, pDvul2 and pRv1 families as probes (Fig. 2).

Probing of pDvul1-1 to genomic DNA digested with *Alu*I, *Hae*III, *Eco*RI, *Bam*HI and *Hind*III detected a strong hybridization smear of a wide molecular weight range (Fig. 2A, lanes 1–2 and 4–6), reflecting a dispersed genomic organization of the pDvul1 family. In addition, the *Rsa*I restriction revealed conserved fragments between 0.2 kb and 1.0 kb in size (Fig. 2A, lane 3). Hybridization to *Msp*I- and *Hpa*II-digested DNA indicated no significant different methylation at CNG sites of the pDvul1 repeat family (Fig. 2A, lanes 7 and 8).

TABLE 1. Major tandemly arranged and dispersed *B. vulgaris* repeats

Repeat	Type	Reference
pEV1	Satellite DNA	Schmidt <i>et al.</i> , 1991
pBV1	Satellite DNA	Schmidt and Metzloff, 1991
pXV	Satellite DNA	Schmidt <i>et al.</i> , 1994
pAV34	Satellite DNA	Dechyeva and Schmidt, 2006
pHC28	Satellite DNA	Schmidt and Heslop-Harrison, 1993
pSV1	Satellite DNA	Schmidt <i>et al.</i> , 1998
pDRV1	Dispersed DNA	Schmidt <i>et al.</i> , 1998
BNR1	Non-LTR retrotransposon, partial	Schmidt <i>et al.</i> , 1995
Tbv1	Ty1-copia retrotransposon, partial	Schmidt <i>et al.</i> , 1995
TPvul1	En/Spm transposon, partial	Jacobs <i>et al.</i> , 2004
Vulmar1	Tc1/mariner transposon	Jacobs <i>et al.</i> , 2004
VulMITE I	Stowaway miniature inverted-repeat transposable element	Menzel <i>et al.</i> , 2006
VulMITE II	Miniature inverted-repeat transposable element	Menzel <i>et al.</i> , 2006
VulMITE III	Miniature inverted-repeat transposable element	Menzel <i>et al.</i> , 2006

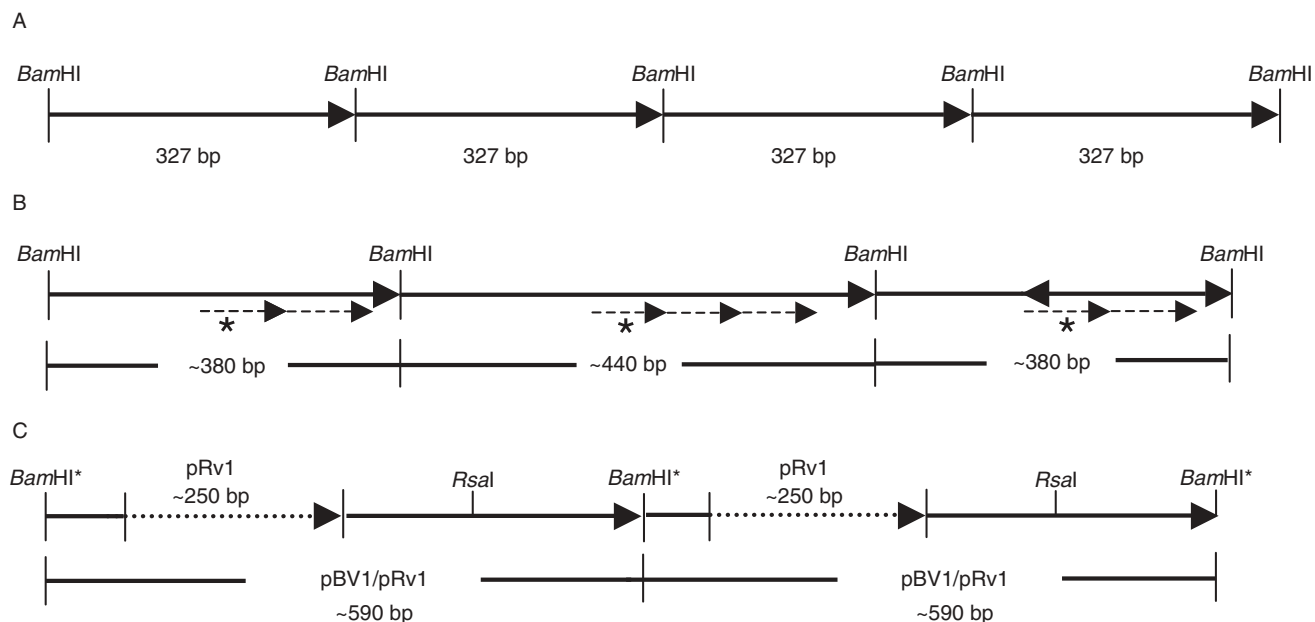


FIG. 1. Schematic representation of the structural variations of the pBV1 satellite repeat in the *B. vulgaris* genome. (A) Tandemly arranged pBV1 monomers of 327 bp. The *Bam*HI restriction sites define the satellite monomers according to Schmidt and Metzloff (1991). (B) Size variations of pBV1 monomers detected on a *B. vulgaris* bacterial artificial chromosome (BAC). The monomer length is increased to approx. 380 bp by an internal duplication (dashed arrow) of a 60-bp sequence motif (dashed arrows, labelled with an asterisk). An amplification of the 60-bp motif resulted in a monomer of approx. 440 bp. (C) Interspersion of a pRv1 unit of approx. 250 bp (dotted arrow) into pBV1 monomers of approx. 340 bp. The combined pBV1/pRv1 unit observed on end-sequenced BACs and plasmid clones is approx. 590 bp in length and contains a unique *Rsa*I restriction site, while the *Bam*HI site is degenerated (indicated by an asterisk).

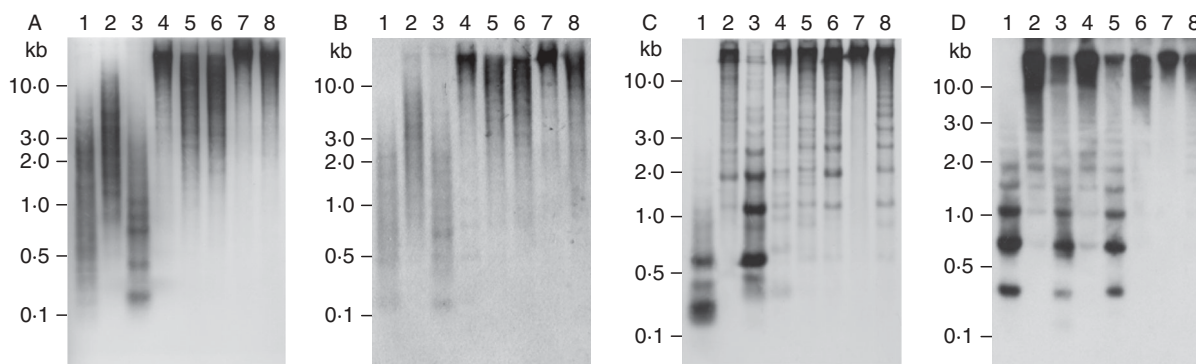


FIG. 2. Southern hybridization of *B. vulgaris* repeats to genomic DNA of the *B. vulgaris* ssp. *vulgaris* cultivar 'KWS 2320', which was digested with *Alu*I (lane 1), *Hae*III (lane 2), *Rsa*I (lane 3), *Eco*RI (lane 4), *Bam*HI (lane 5), *Hind*III (lane 6), *Msp*I (lane 7) and *Hpa*II (lane 8). Blots were separately hybridized with pDvul1 (A), pDvul2 (B) and pRv1 (C). For comparison with the pRv1 pattern, a filter (D) was probed with a monomer sequence of the pBV1 satellite family (Schmidt and Metzloff, 1991).

Hybridization with pDvul2-1 resulted in a pattern of numerous fragments forming a smear, which is distributed over a molecular size range of 0.2 kb to above 10 kb (Fig. 2B, lanes 1–3), or a size above 1.0 kb (Fig. 2B, lanes 4–6). Additionally, in *Rsa*I-digested genomic DNA, several faint, but conserved fragments in a size range of 0.2–1.0 kb are visible (Fig. 2B, lane 3). The hybridization pattern of *Msp*I and *Hpa*II digested genomic DNA suggests no differences of cytosine methylation of the pDvul2 family (Fig. 2B, lanes 7 and 8).

In contrast to the mostly dispersed patterns observed with pDvul1 and pDvul2, hybridization with a 170-bp pRv1-1 fragment produced distinct ladder-like patterns (Fig. 2C,

lanes 1–6 and 8). In *Rsa*I digested genomic DNA, strong hybridization signals consisting of a 0.6-kb monomer band and multimeric bands ranging up to approx. 10 kb (Fig. 2C, lane 3) suggests a satellite-like DNA organization. In contrast, weaker ladder-like banding patterns from 0.6 kb up to above 10 kb are visible in *Hae*III, *Eco*RI, *Bam*HI and *Hind*III restrictions (Fig. 2C, lanes 2–6). Furthermore, two strong *Alu*I bands are localized at 0.2 kb and 0.6 kb, respectively (Fig. 2C, lane 1). Hybridization of *Msp*I and *Hpa*II digests shows significant differences in the cytosine methylation of pRv1 repeats (Fig. 2C, lanes 7 and 8).

Because of the interspersion of pRv1 in the *Bam*HI satellite pBV1, a Southern comparative hybridization with

a pBV1 probe was carried out (Fig. 2D). The *AluI*-, *RsaI*- and *BamHI*-restricted DNA reveals a typical satellite pattern including a monomere of approx. 0.35 kb and the corresponding di- and trimers of 0.75 kb and 1.1 kb. Additionally, presence of weaker signals in the *RsaI* restriction forms an additional ladder with a monomere of 0.65 kb and a dimer of approx. 1 kb. Similar to pRv1, differences in methylation of the cytosine residues were observed by the occurrence of the satellite hybridization pattern only in *HpaII* digested genomic DNA.

Distribution of repeat families within the genus *Beta*

The repeats distribution within the genus *Beta* was analysed by Southern hybridizations with *RsaI*-digested genomic DNA of representative species of the four *Beta* sections *Beta*, *Corollinae*, *Nanae* and *Procumbentes*. Spinach (*Spinacia oleracea*), which also belongs to the subfamily Chenopodiaceae, was chosen as an outgroup species.

Southern hybridization with the dispersed repeat pDvu1 revealed an almost identical hybridization pattern within the section *Beta* (Fig. 3A, lanes 1–8). In species of the section *Corollinae* (Fig. 3A, lanes 9 and 10) the weaker hybridization pattern appears to be almost similar to that in the section *Beta*, with the observed exception of two bands lacking at 0.4 kb and 1 kb, respectively. A similar but weaker pattern is visible in *B. nana* (Fig. 3A, lane 11). Genomic DNA of two species of the section *Procumbentes* (Fig. 3A, lanes 12 and 13) and the outgroup species *S. oleracea* (Fig. 3A, lane 14) shows only a faint hybridization.

Probing of a pDvu2 fragment resulted in a dispersed hybridization pattern (Fig. 3B, lanes 1–14), which appears to be almost similar to the pattern of the pDvu1 repeat family (Fig. 3A, lanes 1–14). Nevertheless, hybridization bands within the species of the four *Beta* sections are more distinct due to reduced background smear (Fig. 3B, lanes 1–11).

The satellite-like hybridization ladder formed by the strong 0.6-kb monomer band specific for the pRv1 family was exclusively identified in species of the section *Beta*. Multimeric bands extending to 3 kb were identified in all species of the section (Fig. 3C, lanes 1–8). Even after an extended time of exposure, no signals were visible in species of the sections *Corollinae*, *Nanae* and *Procumbentes* or in *S. oleracea*.

Chromosomal localization of dispersed repetitive families

The chromosomal organization of the repetitive families pDvu1 and pDvu2 on *B. vulgaris* mitotic chromosomes was analysed by FISH.

Probing of pDvu1 revealed dispersed fluorescent signals with banding of different intensity along all chromosome arms, while terminal euchromatin (indicated by arrows) was largely excluded from hybridization (Fig. 4A).

Successful localization of pDvu2 on sugar beet chromosomes was compromised by its relatively low abundance in the genome. While pDvu1 produced >100 positives on a high-density filter of the plasmid library SGZR00841,

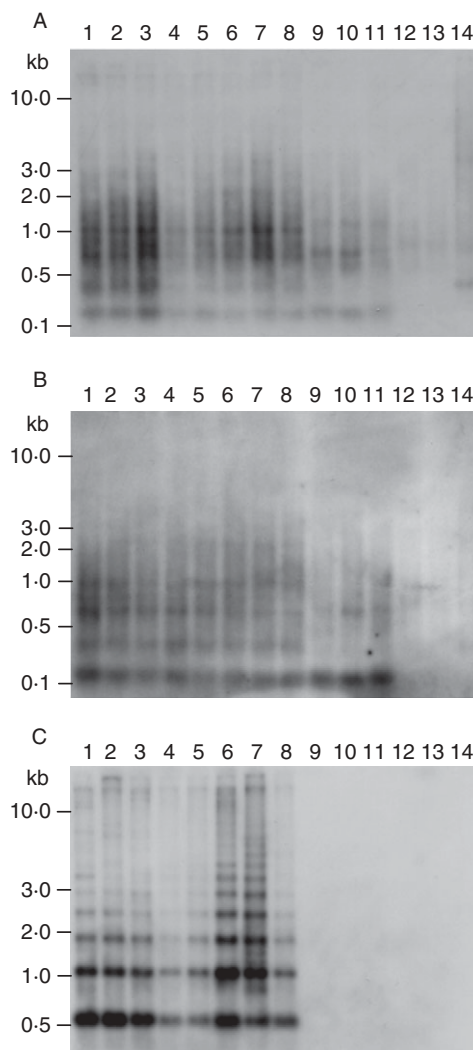


FIG. 3. Species distribution of *B. vulgaris* repeats was analysed by Southern hybridization to *RsaI*-digested genomic DNA of representative species of the four sections of the genus *Beta*. Species of the section *Beta*: sugar beet *B. vulgaris* ssp. *vulgaris* cultivar 'KWS 2320' (lane 1), fodder beet *B. vulgaris* ssp. *vulgaris* cultivar 'Brigadier' (lane 2), garden beet *B. vulgaris* ssp. *vulgaris* cultivar 'Rote Kugel' (lane 3), leaf beet *B. vulgaris* ssp. *vulgaris* cultivar 'Lukullus' (lane 4), *B. vulgaris* ssp. *maritima* (lane 5), *B. vulgaris* ssp. *adanensis* (lane 6), *B. macrocarpa* (lane 7), *B. patula* (lane 8); species of the section *Corollinae*: *B. corolliflora* (lane 9), *B. macrorhiza* (lane 10); species of the section *Nanae*: *B. nana* (lane 11); species of the section *Procumbentes*: *B. procumbens* (lane 12), *B. patellaris* (lane 13); outgroup species: *S. oleracea* (lane 14). Blots were separately hybridized with pDvu1 (A), pDvu2 (B) and pRv1 (C) probes.

there were only ten signals generated by pDvu2. To map pDvu2 physically by FISH, an extended hybridization time, adjusted stringency and signal amplification were applied. The repeat was found on all 18 *B. vulgaris* chromosomes producing doublets of signals of varying intensity. On four chromosomes it was found in the subtelomeric position (Fig. 4B, examples shown by arrows), on two pericentrically (Fig. 4B, arrowheads), and on the remaining chromosomes it was clustered in intercalary chromatin.

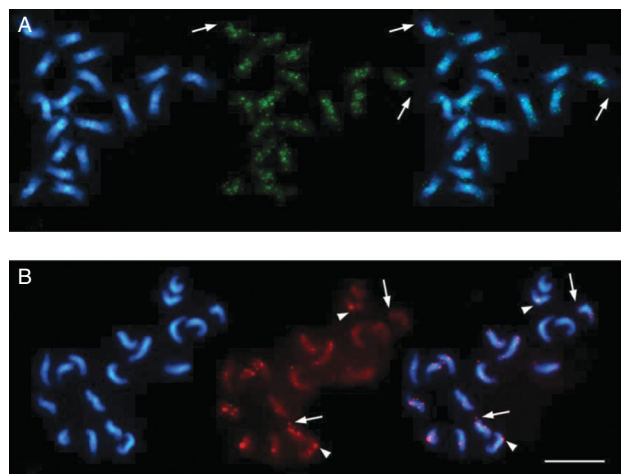


FIG. 4. Fluorescent *in situ* hybridization on *B. vulgaris* mitotic chromosomes. In each panel, the DAPI-stained DNA (blue fluorescence) shows the morphology of the chromosomes. (A) pDvul1-1 (green) is dispersed along heterochromatin of all chromosomes with almost equal signal intensities, except for terminal euchromatin (indicated by arrows). (B) Hybridization with pDvul2-1 (red) revealed doublets of signals of varied strength in terminal (indicated by arrows), centromeric (arrowheads) and intercalary regions of all chromosomes. Scale bar in (B) = 10 μ m.

Centromeric co-localization of pRv1 and the pBV1 satellite family

The pBV1 repeat family, similar to pRv1, was shown to be exclusively present in species of the section *Beta* by Southern hybridization (Schmidt and Metzloff, 1991). FISH on *B. vulgaris* metaphase chromosomes revealed that pBV1 is restricted to the centromeric regions on all 18 sugar beet chromosomes producing signals of different intensity (Fig. 5A, green fluorescence). In contrast, pRv1 hybridized clearly to 12 centromeric regions with signals of variable intensity (Fig. 5A, red fluorescence and overlay). The pRv1 signal strength falls into three classes of strong hybridization on four chromosomes, six centromeres with moderate intensity, and two chromosomes with weak signals (Fig. 5A). Moreover, signals of moderate strength could be confined to the chromosome pair I, which is recognizable by the presence of the terminal nucleolus organizer region (Fig. 5A, arrowed).

The section *Beta* consists of the species *B. vulgaris*, *B. patula* and *B. macrocarpa*. To analyse the centromeric pRv1 and pBV1 organization within the section, comparative FISH on chromosomes of these species was performed. As in *B. vulgaris*, pBV1 hybridization in *B. patula* and *B. macrocarpa* is visible in the centromeric heterochromatin of all 18 chromosomes (Fig. 5C and D, green fluorescence). To accurately compare species-specific pRv1 hybridization patterns, the metaphase chromosomes stained with DAPI as well as the corresponding pRv1 hybridizations were arranged according to the classes of pRv1 signal intensity observed (Fig. 5E). In contrast to the three intensity classes detected on at least 12 *B. vulgaris* chromosomes (Fig. 5E, top), pRv1 hybridization strength in *B. patula* only falls into two classes of four strong and eight moderate centromeric signals, including

the chromosome pair I (Fig. 5E, middle). In *B. macrocarpa*, the pair of chromosome I shows a strong pRv1 hybridization, while the centromeric regions of ten chromosomes reveal only weak signal intensities (Fig. 5E, bottom). FISH on *B. vulgaris* and *B. macrocarpa* show significant differences in hybridization pattern, suggesting a species- and chromosome-specific amplification and organization of pBV1/pRv1 units. Nevertheless, in overlays of pBV1 and pRv1 hybridization signals on metaphase chromosomes of both species, only the centromeric co-localization of both pBV1 and pRv1 signals was detected (Fig. 5A, C, overlays). To achieve higher resolution, pBV1 and pRv1 were hybridized to uncondensed interphase chromosomes of *B. vulgaris* and *B. macrocarpa*. The experiment showed a clear separation pRv1/pBV1 clusters from pBV1 arrays on chromocentres of both species (Fig. 5B, F, arrowed in overlay).

DISCUSSION

Repetitive sequences were estimated to form at least 63 % of the *B. vulgaris* genome (Flavell *et al.*, 1974), with numerous families of repeats discovered by shot-gun cloning or targeted cloning of restriction satellites (Kubis *et al.*, 1998; Dechyeva *et al.*, 2003). Our differential hybridization experiments on high density filters, as well as the bioinformatic analysis of 54684 BAC end sequences indicates that the amount of repetitive DNA in the *B. vulgaris* genome might be even higher than 63 %.

From the fraction of *B. vulgaris* repetitive DNA, the novel families pDvul1, pDvul2 and pRv1 have been identified. Southern analysis with pDvul1 and pDvul2 showed a dispersed genomic organization of both repetitive families that was similar in the sections *Beta*, *Corollinae* and *Nanae*, but differs in the section *Procumbentes*. Such *Procumbentes*-specific absence or low abundance was also observed for *Beta* repeat families such as pHC, pRN, pSV and pDRV (Schmidt and Heslop-Harrison, 1993; Kubis *et al.*, 1997; Schmidt *et al.*, 1998). These results support the suggestion based on the analyses of ITS1 sequences, that species of *Procumbentes* might form a separate genus designated *Patellifolia* (Kadereit *et al.*, 2006). In FISH on *B. vulgaris* metaphase chromosomes, pDvul1 is widely scattered including centromeres, while pDvul2 produced few pairs of signals dispersed in intercalary and terminal chromatin, indicating that pDvul2 is less abundant. A weak ladder-like pattern superimposed on a smear and additional irregular bands observed in Southern hybridization suggests an organization of pDvul2 in dispersed clusters of presumably small tandem arrays. Similarly, the *AluI* repeat pAp22 from the wild beet *B. procumbens* is dispersed with local amplification over the chromosomes, but is more abundant (Dechyeva *et al.*, 2003). Dispersion along chromosomes is also typical for some Ty1-copia retrotransposons and LINEs (Katsiotis *et al.*, 1995; Schmidt *et al.*, 1995; Heslop-Harrison *et al.*, 1997). Retrotransposons and transposons as highly amplified components of plant genomes play an important role in the evolution of the genome structure and function (Kumar and Bennetzen, 1999) and are a source of dispersed

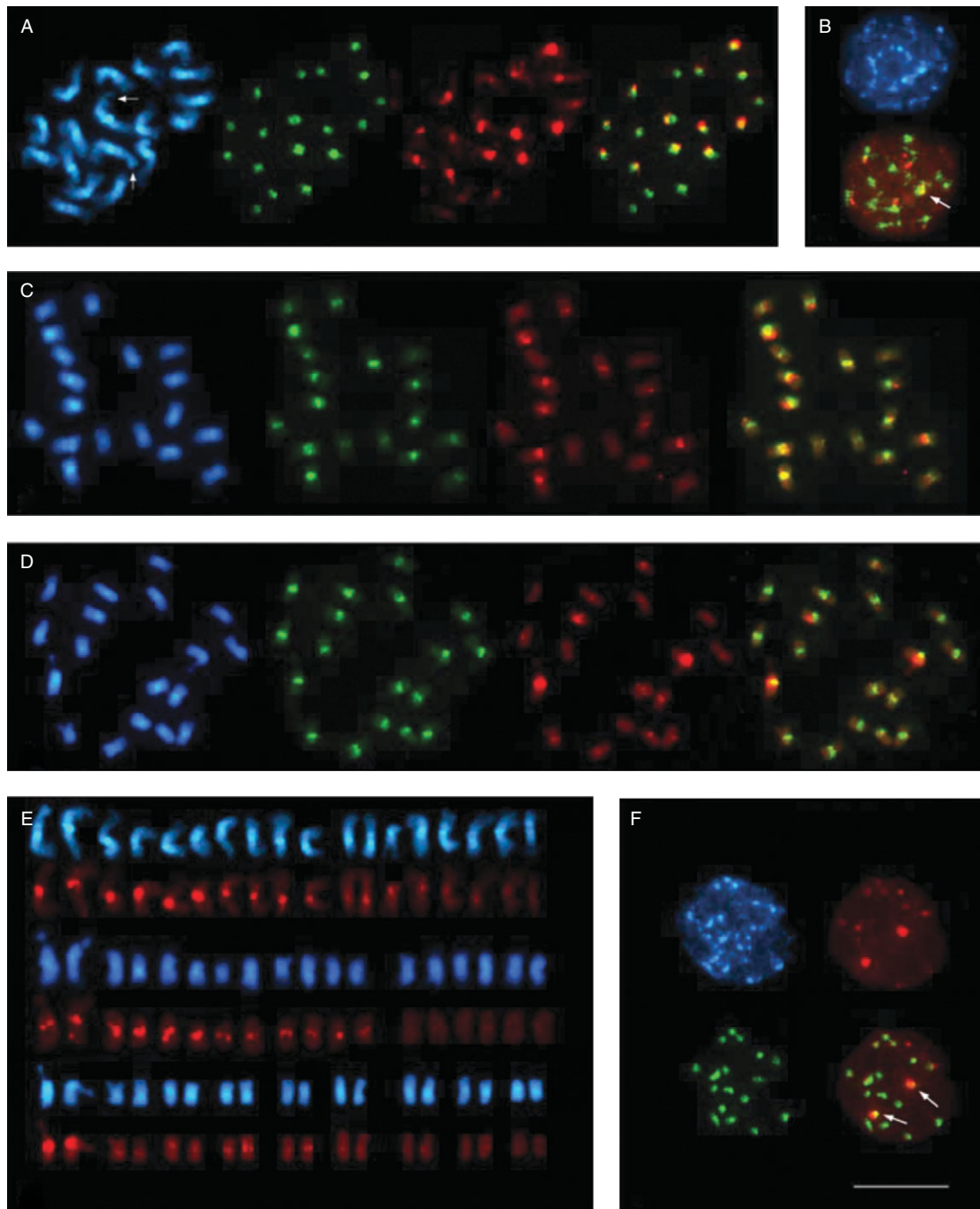


FIG. 5. Fluorescent *in situ* hybridization to metaphase and interphase chromosomes of species of the section *Beta*. In each panel, the DAPI-stained DNA (blue fluorescence) shows the morphology of the chromosomes. The pBV1 hybridization signals are visible as green fluorescent signals, and red fluorescence indicates pRv1 hybridization. Comparison of the signal pattern of pBV1 and pRv1 probes hybridized to metaphase chromosomes of *B. vulgaris* (A), *B. patula* (C) and *B. macrocarpa* (D), indicating a species-specific centromeric localization of both repeat families. The *B. vulgaris* chromosome pair I is marked by arrows. The right picture in (A), (C) and (D) represents an overlay of the pBV1 and pRv1 hybridization. (B) Centromeric localization of pBV1 and pRv1 is visible as signals on chromocentres of *B. vulgaris* interphase nuclei. The embedment of pRv1 into areas of pBV1 is highlighted by an arrow in the overlay (bottom). (E) Arrangement of metaphase chromosomes from *B. vulgaris* (top), *B. patula* (middle) and *B. macrocarpa* (bottom) according to the observed signal strength of centromeric pRv1 hybridization. The pair of chromosome I for each species is placed on the left. (F) Centromeric localization of pBV1 and pRv1 signals in the centromeric heterochromatin of *B. macrocarpa* interphase chromosomes. The distinct cluster of pRv1 adjacent to a pBV1 area is visible as yellow fluorescence (indicated by arrows, bottom right). Scale bar (in F) = 10 μ m.

sequence families. In barley, dispersed repeats were identified as remnants of retrotransposon-specific reverse transcriptase fragments (Liu and Somerville, 1996).

Transductions of genomic sequences by plant retroelements have been demonstrated for Bs1 from maize (Jin and Bennetzen, 1994), and the dispersed repeats pAp4 and

pAp22 from *B. procumbens* are directly linked to Ty1- *copia* or *env*-like retrotransposons (Dechyeva *et al.*, 2003). It is, however, unclear whether and by which mechanism mobile elements might have participated in the amplification of pDvul2 repeats. In fact, the repetitive families pDvul1 and pDvul2 exhibit no sequence homology to EMBL entries of conserved retrotransposon or transposon genes. Thus, pDvul1 and pDvul2 might be derivatives of untranslated transposon regions, including long terminal repeats, or of yet undiscovered *B. vulgaris* transposable elements. Otherwise, they might have an independent origin. Such dispersed repetitive families with no relationship to retrotransposon-like sequences have been observed in barley, rice and tobacco (Hueros *et al.*, 1993; Kiefer-Meyer *et al.*, 1996; Horakova and Fajkus, 2000).

Satellite DNA sequences comprise a large proportion of the repetitive plant genomic DNA. The differential hybridization presented in this work revealed that the majority of the previously described repetitive families belong to the group of satellite DNA. So far, 14 different satellite DNA families from the four sections of the genus *Beta* have been characterized (Dechyeva *et al.*, 2003; Dechyeva and Schmidt, 2006). Most of these repetitive sequences were identified as restriction satellites with monomers falling into size classes of approx. 150–180 bp and 300–360 bp. The majority of the cloned *Beta* satellite monomers can be assigned to the group of 150- to 160-bp repeats, which presumably corresponds to the stretch of nuclear DNA wrapped around a single nucleosome core (Fischer *et al.*, 1994; Vershinin and Heslop-Harrison, 1998; Heslop-Harrison, 2000). This length of satellite motifs may be favourable for chromatin packaging, thus undergoing selection resulting in accumulation of repeats of this size over evolutionary time scales. As satellite DNA represents a fast-evolving portion of plant genomes, the formation of larger and often complex repeats, including dimerization, is typical of satellite families and has been observed in many plant species (Ingham *et al.*, 1993; Grebenstein *et al.*, 1996; Simoens *et al.*, 1988; Dechyeva *et al.*, 2006). Among the *Beta* tandem repeats, the pRN1 satellite from *B. corolliflora* shows an unusual length of 202–233 bp (Kubis *et al.*, 1997), and the 240 bp *AluI* satellite pAp11 from *B. procumbens* (Dechyeva *et al.*, 2003) represents one and a half 160-bp monomers as a whole unit. By computational analyses of 27 342 end-sequenced *B. vulgaris* BACs, distinct variants have been observed with structural changes of the *B. vulgaris* satellite repeat pBV1, which was initially described as a *Bam*HI satellite with a monomer size of 327 bp (Schmidt and Metzloff, 1991). After the duplication of an internal 60-bp fragment, the pBV1 monomer size has increased up to 380 bp. Moreover, amplification of the 60-bp motif results in a pBV1 monomer unit of 440 bp. Tandemly arranged plant repetitive DNA often contains subrepeats of 60 bp, which are themselves built by two units of approx. 30 bp (Ingham *et al.*, 1993). Such duplication was also described as the origin of the 60-bp subrepeat of pBV1 (Schmidt and Metzloff, 1991). Thus, the stepwise duplication and subsequent amplification of short sequence motifs

might be a common mechanism for the generation of plant satellite DNA.

Large arrays of tandemly repeated satellite DNA localized in centromeric heterochromatin are assumed to substantially contribute to centromere function, e.g. segregation of sister chromatids during mitosis and meiosis, by an interaction with the kinetochore protein CENH3 (Houben and Schubert, 2003). Despite their conserved function, centromeric satellites evolve more rapidly than other genomic sequences (Hall *et al.* 2003), most likely by the genetic mechanisms of unequal crossing over, replication slippage and unequal sister chromatid exchange (Smith, 1976; Dover, 1982). As a result, sequences of centromeric satellite repeats vary even between closely related species, often resulting in new centromer-specific satellite variations taking over centromere function (Ma *et al.*, 2007). Thus, the localization of the satellite pBV1 in the centromeric heterochromatin on all chromosomes exclusively observed in the three species of the section *Beta* might be an indication of a section-specific role for pBV1 satellites in centromere function, although there is no experimental evidence for this assumption so far.

Beside the structural variations of the pBV1 satellite monomer, Southern hybridization experiments of *RsaI*-digested genomic *B. vulgaris* DNA probed with the pRv1 repeat revealed the amplification of a complex satellite unit composed of pBV1 and pRv1 units. In plants, insects and humans, tandem repeats were described representing combinations of closely related satellite subrepeats (Grebenstein *et al.*, 1996; Pons *et al.*, 2003; Rudd and Willard, 2004; Dechyeva and Schmidt, 2006). In contrast to pBV1, no pRv1 multimers were identified by sequence analysis of BAC ends and plasmid clones. Moreover, no amplification of tandemly arranged pRv1 di- and multimeric products was observed in PCR analyses using pRv1-specific primers (data not shown). Therefore, the existence of pRv1 as an independent, separate *Beta* satellite can largely be excluded.

The chromosome specificity of centromeric satellite DNA has been observed only in few plant species like *Brassica*, sugarcane, *Arabidopsis* and the wild beet *B. procumbens* (Harrison and Heslop-Harrison, 1995; Nagaki *et al.*, 1998; Kawabe and Nasuda, 2005; Dechyeva *et al.*, 2003). In the three species of the section *Beta*, the interspersion of the complex pBV1/pRv1 with pBV1 satellite arrays was detected by FISH in the centromeric heterochromatin of up to 12 out of 18 chromosomes. The presence of pBV1/pRv1 arrays on the remaining three chromosome pairs is unlikely due to the conditions used in FISH. The high degree of sequence identity in the *B. vulgaris*-specific pBV1/pRv1 units analysed suggests a strong homogenization process during amplification, which is comparable to the homogenization of higher order satellite structures observed in humans and insects (Rudd and Willard, 2004; Kuhn *et al.*, 2008). Therefore, a loss of pBV1/pRv1 arrays, which might have been present on all chromosomes of a progenitor of *Beta* section species, is unlikely.

The comparison of the species-specific pRv1 FISH signal intensities indicates a remarkable chromosome-specific amplification of pBV1/pRv1 units in the cultivated species *B. vulgaris*. It would be interesting to analyse the physical organization of pBV1/pRv1 units in an ancestor of cultivated beets, like the wild beet *B. maritima*, to unravel whether the observed dynamics of centromeric pBV1 satellite amplification has occurred during domestication of sugar beet, which started in the late 18th century (Fischer, 1989).

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