Theor Appl Genet (1998) 97: 696–704

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Molecular and chromosomal organization of two repetitive DNA sequences with intercalary locations in sugar beet and other *Beta* species

Received: 18 March 1998 / Accepted: 31 March 1998

Abstract In a search for repetitive DNA sequences in the sugar beet genome, two sequences with repeat unit lengths of 143 and 434 bp were isolated and characterized. The pSV family showed an unusual conservation of restriction sites reflecting homogenization of the analyzed repeats. Members of the family are organized as tandem repeats as revealed by PCR and sequencing of dimeric units. The pSV satellite occurs in large intercalary arrays which are present on all chromosome arms of sugar beet. The pSV sequence family is present in different abundance in the sections *Beta*, *Corollinae* and *Nanae* but is not detectable by Southern hybridization in the section *Procumbentes*. The pDRV family is characterized by an interspersed genomic organization. The sequence is detectable in all sections of the genus and is amplified in species of the section *Beta* but was also detected, although at lower abundance, in the remaining three sections. Fluorescent in situ hybridization has shown that the pDRV sequence family is dispersed over all chromosomes of the sugar beet complement with some regions of clustering and centromeric depletion.

Key words Satellite DNA · Repetitive DNA · Fluorescent in situ hybridization · *Beta vulgaris* · Dispersed repeats

Communicated by F. Mechelke

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Introduction

Nuclear genomes of higher plants vary enormously in size. Some of this variation is due to polyploidy, but even among diploids which have similar numbers of genes, the total amount of nuclear DNA ranges from below 150 Mbp in species such as horse chestnut, rosaceous species and *Arabidopsis thaliana*, to more than 20000 Mbp in pines. These differences in genome size include differences in repetitive DNA sequences which normally represent the majority of a genome.

Beta vulgaris L. is a valuable model species for investigating the large-scale organization of the nuclear genome and the molecular and chromosomal organization of repetitive DNA because it is diploid ($2n = 18$) and has a relatively small genome of 758 Mbp (Arumuganathan and Earle 1991). Cultivars of *B. vulgaris* (sugar beet, fodder beet, beet root and mangold) are crops grown in temperate climates for sugar production, animal feed or as vegetables. Along with some wild beet species, they belong to the section *Beta* of the genus *Beta* (Chenopodiaceae). The genus *Beta*, with four sections (*Beta*, *Corollinae*, *Nanae* and *Procumbentes*) and about 20 diverse species, provides a group of closely and more distantly related species which are useful for studies of sequence and genome evolution (Barocka 1985). A systematic search of *Beta* genomes has revealed about ten major families of non-homologous repetitive DNA sequences (Schmidt et al. 1991; Schmidt and Heslop-Harrison 1993, 1996; Schmidt et al. 1995; Kubis et al. 1997). These have been extensively characterized at the molecular level by a combination of sequencing, conventional or pulsed field gel

electrophoresis and Southern hybridization, as well as by in situ hybridization. In particular, fluorescent in situ hybridization (FISH) is a valuable and accurate method for the physical localization of repetitive sequences along chromosomes. Like most plant species, the majority of the sugar beet genome consists of repetitive DNA, consisting typically of sequence motifs from 2 bp up to several kb which are reiterated up to many thousands of times within the genome. Repetitive DNA can be assigned to several main sequence classes which differ in their organization along and their localization in the chromosomes, although intermediate forms of organization also exist.

Tandemly repeated DNA is characterized by repeating units which are arranged adjacently to each other; examples of tandemly repeated sequences are satellites, microsatellites, or gene units encoding ribosomal RNAs. DNA families consisting of tandem repeats constitute large blocks of chromatin which do not decondense at interphase as shown by fluorescent in situ hybridization in many plant species. They represent a large proportion of the heterochromatin and occur at characteristic regions of many or all chromosomes of the genome. The region around the centromere of plant chromosomes are sites where satellite DNA families are preferentially located, and tandem arrays have been reported, for example, in many cruciferous species (Maluzynska and Heslop-Harrison 1991; Harrison and Heslop-Harrison 1995; Kamm et al. 1995; Grellet et al. 1986), in *Vignia unguiculata* (Galasso et al. 1995) and *Pennisetum glaucum* (Kamm et al. 1994). Satellite families occur also in close proximity to the physical ends of chromosomes. These subterminal satellite sequences have been reported from cereal (Brandes et al. 1995; Vershinin et al. 1995) and *Allium* species (Barnes et al. 1985; Pich et al. 1996). In contrast to satellite arrays at centromeric and subtelomeric regions, only a few intercalary satellite DNA families have been found, mostly in smaller arrays, forming multiple bands along chromosomes and sometimes co-localizing with C-bands, like the sequence pSc119.2 within the wheat B genome (Mukai et al. 1992). Intercalary C-bands may include microsatellite sequences like GAA, as was shown in *Hordeum vulgare* (Pedersen and Linde-Laursen 1994). The sequence pHcKB6, isolated from *Hordeum chilense*, also hybridizes to multiple intercalary sites on chromosomes from many *Hordeum*, *Aegilops* and *Triticum* genomes (Anamthawat-Jonsson and Heslop-Harrison 1993; Nagaki et al. 1995).

In contrast to tandemly repeated sequences, dispersed repetitive DNA sequences are much more variable. A large fraction of dispersed plant sequences originates from families of mobile DNA elements comprising transposons and retrotransposons (Kunze et al. 1997). They are scattered throughout the genome, interspersed with themselves and other sequences, and distributed over all or most chromosomes of a complement often showing site-specific amplification or depletion (Heslop-Harrison et al. 1997).

It is clear that there are many dispersed sequences with unknown amplification and dispersion mechanisms within the plant genome. Dispersed repeats have been found in many plants including rice (Kiefer-Meyer et al. 1996), cotton (Zhao et al. 1995) and *Brassica* species (Kapila et al. 1996). Genomic in situ hybridization (GISH) is able to discriminate between related genomes of hybrid origin plants, because of the uniform nature of the chromosome labelling, with little or no difference in regions known to include tandem repeats, this must depend on the hybridization of families of dispersed repetitive sequences with genome specificity.

We have aimed to search for major repetitive DNA sequences in *Beta* genomes to provide a model of the sugar beet genome which is useful for marker-assisted selection, gene isolation and transfer, and the investigation of plant genome evolution and structure. In this study, we describe two families of repeated DNA sequences which contrast in their genomic and chromosomal organization but contribute to the repetitive DNA of the *B. vulgaris* genome.

Materials and methods

Plant material and DNA isolation

Seeds from wild beet species of all four sections of the genus *Beta* were provided by Dr. L. Frese (Institut für Pflanzenbau der Bundesforschungsanstalt für Landwirtschaft, Braunschweig, Germany) and by Dr. B. Ford-Lloyd (University of Birmingham). The *Beta* accession 'Ames 4527' was received as *Beta trigyna*. However, despite morphological similarities, cytological studies showed it to have $2n = 36$ chromosomes, a number not characteristic for *B. trigyna* ($2n = 45$ or 54). Seeds from cultivars of *B. vulgaris* were obtained commercially. Genomic DNA was isolated from fresh leaf material of greenhouse-grown plants as described by Schmidt and Heslop-Harrison (1993).

Molecular analyses

Clones were selected from genomic plasmid libraries of *B. vulgaris* 'Rosamona' constructed to isolate tandemly repeated DNA and retroelement-like sequences (Schmidt et al. 1991; Kubis et al. 1998) and sequenced on an automated DNA sequencer (LICOR). According to the conservation of restriction sites or genome organization, clones were designated pSV (*Sau*3AI satellite from *B. vulgaris*) and pDRV (dispersed repeat from *B. vulgaris*), respectively. For investigation of the genomic organization of pSV repeats, PCR of *B. vulgaris* DNA was conducted for 35 cycles (1 min 94°C, 1 min 43°C, 1 min 72°C) using primers P1 5′-CTCATCTTTGGTGTGGTTG-3′ and P2 5′-CACTTGGTTCAATATAGGGC-3′. PCR products corresponding to dimers of pSV were excised from agarose gels, cloned using a TA-Cloning kit (Invitrogen) and sequenced. For Southern experiments, genomic DNA of *Beta* species was digested with different restriction enzymes, transferred onto nylon membranes, and hybridized either radioactively with ^{32}P-dCTP or non-radioactively using the ECL system (Amersham).

Chromosome preparation and fluorescent in situ hybridization

Chromosome spreads were made from root-tip meristems of *B. vulgaris* 'Rosamona' as described (Schmidt et al. 1994). Cloned probes were labelled with digoxigenin-11-dUTP by PCR. Pre-treatment of chromosomes, fluorescent in situ hybridization, and detection of hybridization sites were performed according to Schmidt and Heslop-Harrison (1996) except that chromosomes were also counterstained with propidium iodide. After FISH, chromosomes were examined by UV microscopy using different filter sets, and photomicrographs were taken on Fujicolor Super HG 400 print film. Negatives were digitized on a Nikon film scanner, and processed and printed with Adobe Photoshop software using only functions affecting the whole image equally.

Results

Among the clones of repetitive DNA sequence which were isolated from sugar beet (Schmidt et al. 1991; Kubis et al. 1997), members of two families, not homologous to each other or any reported DNA sequence in the EMBL-Genbank, were sequenced (Fig. 1 A, B). The pSV family was characterized as a restriction satellite defined by conserved *Eco*RI, *Rsa*I or *Sau*3AI sites each separated by 143 bp and an AT-content of about 57%. pDRV was longer (163–434 bp) and 70% AT-rich with no strand asymmetry. The motif TTTAAA (the *Dra*I recognition site) occurred eight times within the sequence pDRV1 and represented the majority of short sub-repeats.

Southern hybridization of pSV and pDRV probes to genomic digests of *Beta* species from all four sections of the genus showed that both families were abundant in all species of the section *Beta*, with few major polymorphisms, but less abundant in the sections *Corollinae* and *Nanae* (Fig. 2). pDRV was detectable by weak hybridization signals in the section *Procumbentes*, while the pSV family was not detected. Both the pattern and strength of hybridization of the two sequences indicated that the *Beta nana* genome is more similar to species in the section *Corollinae* than those in the section *Beta*. The pSV family gave additional information about the relationships of species in the section *Corollinae*. *Beta corolliflora* and *B. trigyna* shared the major restriction fragments with species in the section *Beta*, while *Beta lomatagona* (like *Beta nana*) had only a larger restriction fragment. The hexaploid species *Beta intermedia* ($2n = 6x = 54$) shared major bands with both these groups, supporting the morphological evidence for its intermediate position.

In the section *Beta*, both repetitive DNA families were characterized by abundant restriction fragments corresponding in length to the repeating unit (Figs. 1 and 2, lanes 1–11). There was no evidence for a ladder of hybridization fragments, as found with many tandemly organized repetitive sequences, where variability in restriction sites (or in methylation) gives a range of multimers. With pSV, the repeat units were concentrated in a few discrete restriction fragments, mostly monomers and a much weaker band of twice the length, indicating high conservation of the restriction sites, as also suggested by the site conservation seen in the sequences of all seven units (Fig. 1). Evidence for divergence, but strong (and in evolutionary terms rapid) homogenization of the sequence family, comes from a comparison of the *Sau*3AI and *Eco*RI digests hybridized with the pSV sequence. Outside the section *Beta*, no 143 bp units defined by *Sau*3AI were detected, whereas they were detected with *Eco*RI in some species of section *Corollinae*. The pSV family is represented by larger restriction fragments in digests with these and eight additional enzymes (Fig. 3 A), indicating its involvement in higher-order structures.

For further investigation of the genomic organization of the pSV family, primers (Fig. 1) were constructed to investigate larger structures from genomic DNA (Fig. 3 B). The PCR product showed a regular ladder pattern with fragments of about 143 bp, 246 bp, 429 bp and longer. Digestion with enzymes revealing the monomers, namely *Rsa*I, *Sau*3AI and *Eco*RI (Figs. 2 A, 2 B, 3 A), cut the PCR products to give only the 143-bp unit. These results provide evidence that the repeat units were organized in tandem arrays. Single nucleotide changes in the sequence (Fig. 1 A) could generate *Alu*I, *Hae*III and *Hin*fI recognition sites, so the lack of digestion with these enzymes and complete digests with *Rsa*I, *Sau*3AI and *Eco*RI, provide further evidence for the homogeneous nature of the pSV sequence.

In situ hybridization of pSV5 to metaphase chromosomes showed that it is present in large arrays at intercalary sites on most chromosome arms but with variable abundance (Fig. 4 A). On extended prometaphase chromosomes (Fig. 4 B), sites were single with no interruptions of non-homologous sequences, and co-localization with conspicuous DAPI-staining bands was evident. Hybridization of pSV5 to interphase nuclei showed numerous punctate hybridization sites over the area of the nucleus with the exception of the nucleolus (Fig. 4 C); no clustering of sites in nuclear domains or sectors was observed. In situ hybridization of pDRV1 shows that the sequence is distributed on all 18 chromosomes, but excluded from the DAPI-staining centromeric regions and from subtelomeric regions (Fig. 4 D). Discrete clusters of the sequence were detected at intercalary sites, and there were differences between chromosome arms in both the overall strength and distribution of the hybridization signal.

Discussion

The analysis of two highly repetitive DNA sequences enabled us to investigate aspects of both sequence and species evolution and relationships. Satellite DNA is a sequence class which allows interesting insights into the evolution of sequence families, chromosomes and

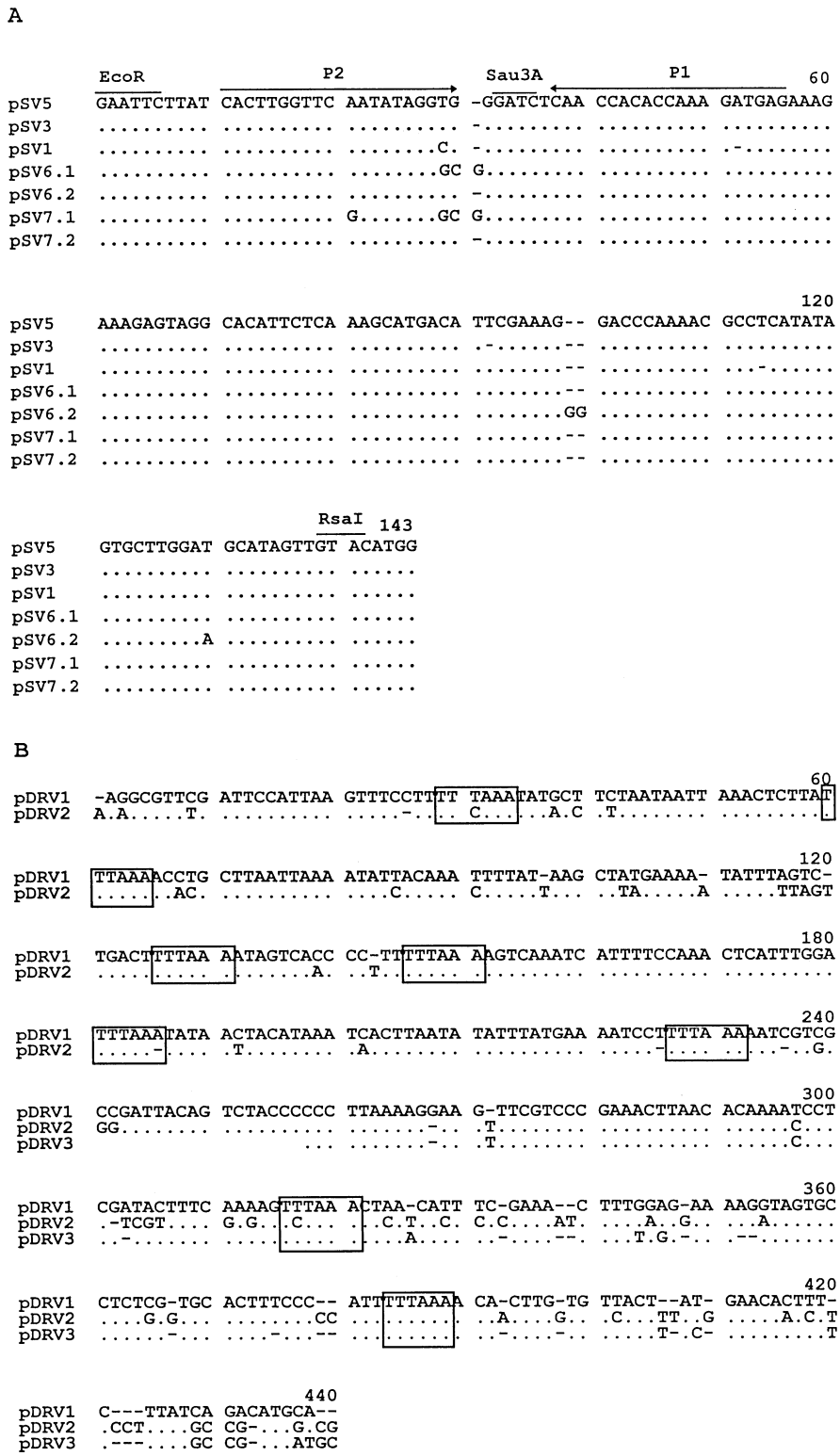

Fig. 1A The sequences of several pSV single units and two dimers, pSV6 (*pSV6.1* and *pSV6.2*) and pSV7 (*pSV7.1* and *pSV7.2*). The positions of PCR primers are shown by *arrows*; conserved recognition sites for *Eco*RI, *Sau*3AI and *Rsa*I are indicated. **B** Sequence of three repeating units of pDRV. Short TTTAAA repeats (*Dra*I recognition sites) are *boxed*. Only nucleotide changes are given; *dots* indicate identical nucleotides, and *dashes* were introduced for maximal alignment. Sequences have been deposited in the EMBL database (pSV5 = Z75011, pDRV1 = Z69351, pDRV2 = Z69352, pDRV3 = Z69353)

genomes, as well as the phylogeny of species (Hemleben et al. 1992; Kamm et al. 1995).

The taxonomy of species within the section *Corollinae* is complicated, and this section can be considered as a complex of wild beets with ongoing speciation, interspecific hybridization, and a high degree of genetic variability. The relationship between the tetraploid species *B. corolliflora* and the diploid *B. lomatogona* and

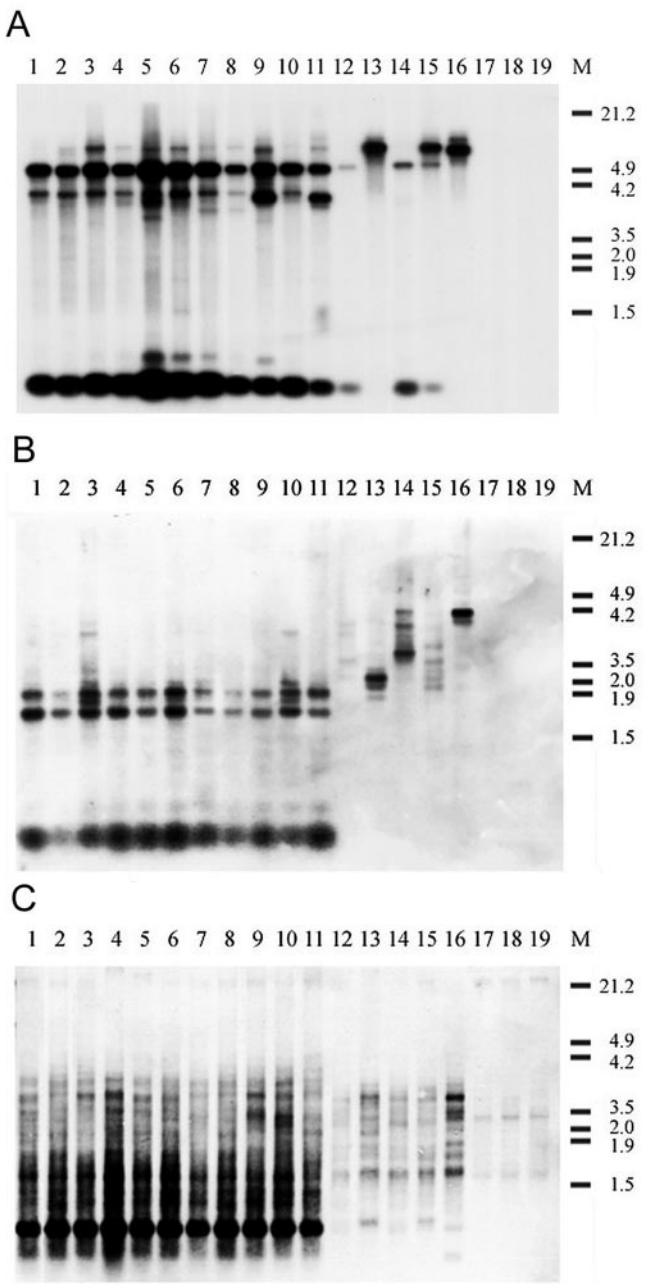

Fig. 2 Genomic organization of the repetitive sequence families in restriction enzyme digests of *Beta* species from all four sections with (**A**) *Eco*RI, (**B**) *Sau*3AI and (**C**) *Hin*fI. Southern blots were hybridized with pSV5 (**A, B**) and pDRV1 (**C**) Cultivars (1–4) and wild species of the sections *Beta* (5–11), *Corollinae* (12–15), *Nanae* (16) and *Procumbentes* (17–19) included are: (*1*) *B. vulgaris* 'Zulu', (*2*) *B. vulgaris* 'Rosamona', (*3*) *B. vulgaris* (beet root 'Monogram'), (*4*) *B. vulgaris* (mangold 'Lukullus'), (*5*) *B. cicla* (BGRC 56736), (*6*) *B. maritima* (BGRC 56651), (*7*) *B. maritima* (BGRC 61198), (*8*) *B. trojana* (BGRC 61212), (*9*) *B. macrocarpa* (BGRC 35276), (*10*) *B. adanensis* (BGRC 32379), (*11*) *B. patula* (BGRC 56782), (*12*) *B. corolliflora* (PI 264352) (*13*) *B. lomatogona* (BGRC 17831), (*14*) *Beta* ssp. (Ames 4527), (*15*) *B. intermedia* (BGRC 17919), (*16*) *B. nana* (81FD26), (*17*) *B. procumbens* (BGRC 35336), (*18*) *B. webbiana* (BGRC 56685), and (*19*) *B. patellaris* (BGRC 57667). Molecular-weight markers (*M*) are given in kb

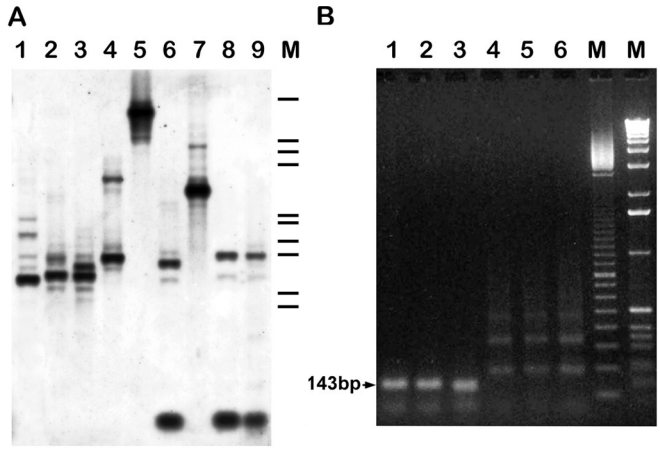

Fig. 3 A Genomic digests of sugar beet DNA with nine different enzymes (left to right, *Dde*I, *Hin*fI, *Hae*III, *Alu*I, *Dra*I, *Rsa*I, *Bam*HI, *Nde*II, *Sau*3AI) showing the conservation of most units of the pSV family after probing with pSV1. Monomers are only detected in digests with *Rsa*I, *Nde*II and *Sau*3AI. Enzymes were chosen such that a single nucleotide exchange would generate recognition sites within the repeats (except *Dra*I). **B** PCR of genomic sugar beet DNA with primers P1 and P2 generates a ladder-like amplification product typical for satellite DNAs. Restriction of the PCR product with *Sau*3AI (*lane 1*) , *Eco*RI (*2*) and *Rsa*I (*3*) reveals monomers showing conservation of recognition sites, while digestion with *Hae*III (*4*), *Hin*fI (*5*) and *Alu*I (*6*) do not cut, indicating that these sites are not generated by the divergence of satellite repeats. DNA size markers (*M*) are lambda/*Eco*RI + *Hin*dIII **(A)** and a 100 bp-ladder (**B**) (left) and a 1 kb-ladder (right)

Beta macrorhiza (not investigated here) are relatively clear, and they are considered as basic species of the *Corollinae*. Other wild beets of the section, including *B. trigyna* and *B. intermedia*, are poorly defined and show variable ploidy levels often involving interspecific hybridization (Reamon-Büttner et al. 1996). Apomixis is widely distributed in this section, and interspecific hybridization is common since most *Corollinae* species colonize the same ecological habitats. Hence, the exact taxonomic classification of species and their relationship often remain uncertain. Indeed, molecular cytogenetic studies of *B. trigyna* 'Ames 4527', conducted during the course of this work, have shown that this *Beta* accession has 36 chromosomes, a number which is characteristic for *B. corolliflora*. However, unequivocal allocation to *B. corolliflora* is not possible because both the number and chromosomal position of 5S rRNA genes is different from all *B. corolliflora* investigated so far (Steffensen 1997). The results from Southern hybridization with pSV and pDRV clones indicate that *B. corolliflora* may have played a role in the generation of *B. trigyna* 'Ames 4527'.

Interestingly, *B. nana* and *B. lomatogona* showed a similar pattern after hybridization of pSV and pDRV to genomic DNA digested with *Eco*RI and *Hin*fI, respectively. This is in agreement with taxonomic suggestions grouping *B. nana* in close relationship to the section *Corollinae* (Barocka 1985). Although

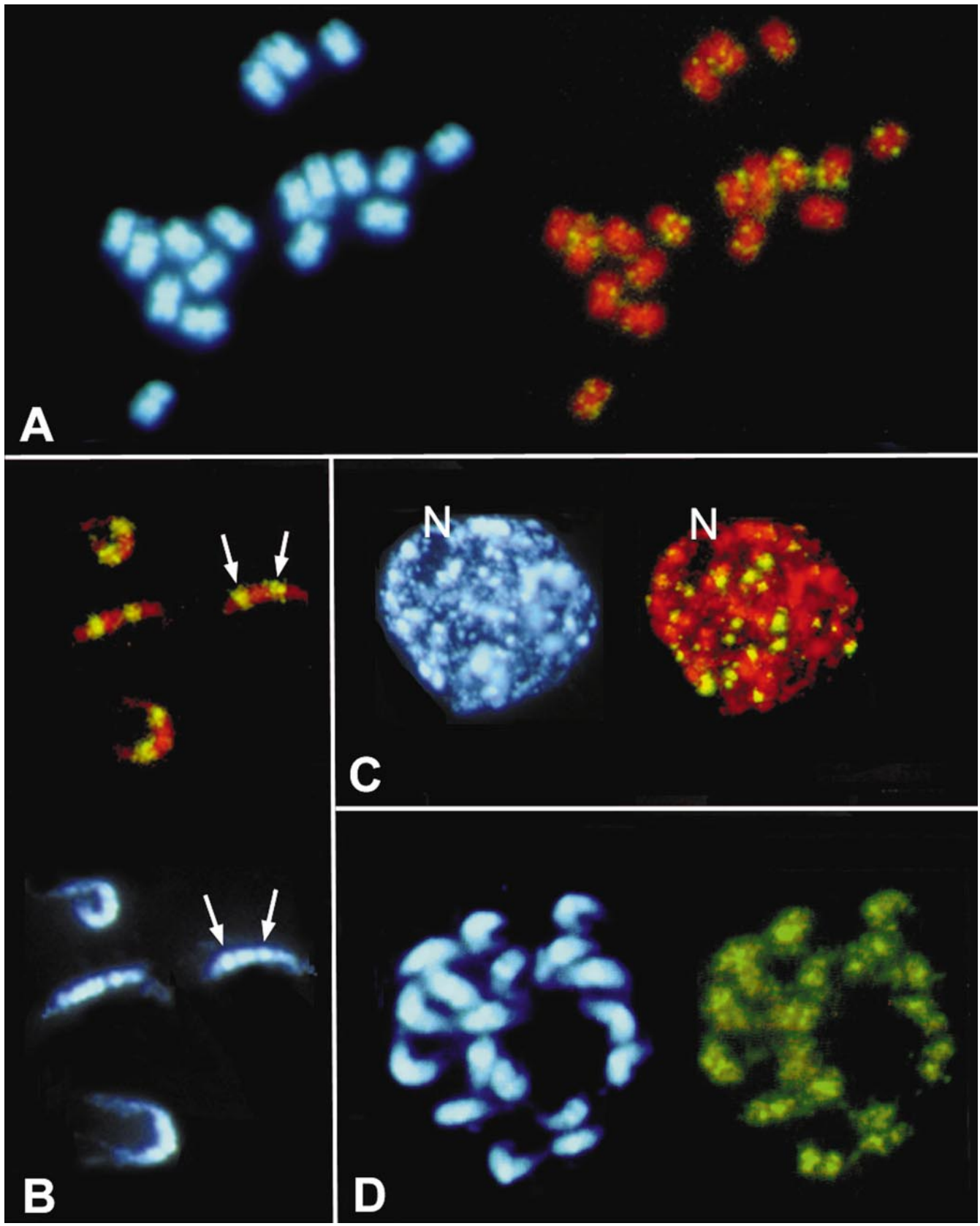

Fig. 4 In situ hybridization of both pSV1 (**A, B, C**) and pDRV1 (**D**) to chromosomes of *B. vulgaris* 'Rosamona' at interphase, prophase and metaphase. *Blue fluorescence* in each panel shows DAPI staining. *Orange-red fluorescence* (**A, B, C**) is from additional counterstaining of chromosomes with propidium iodide. The *arrows* (**B**) point to examples of the conspicuous knob of intercalary chromatin, where the pSV satellite is largely localized. *N* indicates the nucleolus

the existence or absence of satellite families can support the interpretation of species relationships, any taxonomic conclusion based on molecular data of repetitive DNA needs careful consideration: molecular data can be used to support interpretations of species relationships but should be tested on a large sample of accessions. It cannot be ruled out that interspecific hybridization, as occuring in the section *Corollinae*, may result in genomic rearrangements, amplification or deletion of repetitive DNA in hybrids as observed in other genera (Kamm et al. 1995; Wendel et al. 1995).

The pSV family of satellite repeats is located on both arms of sugar beet chromosomes in an intercalary block of DAPI-positive DNA which is clearly detectable at prophase (Fig. 4 B). The organization of satellite repeats in large arrays at intercalary positions is relatively infrequently observed in plants but is a characteristic feature of the chromosome structure in sugar beet (Schmidt and Heslop-Harrison 1998). Typically, satellite DNA arrays are present around the centromeres or in subterminal regions (Kamm et al. 1994; Harrison and Heslop-Harrison 1995; Vershinin et al. 1995; Pich et al. 1996). The conspicuous DAPI-positive region of sugar beet chromosomes harbours several other non-homologous satellite DNA families (Schmidt and Heslop-Harrison 1993, 1998; Kubis et al. 1997) and also clusters of LINEs (Schmidt et al. 1995) suggesting that a large proportion of the repetitive DNA of the sugar beet genome is concentrated in a few intercalary regions. The pSV family adds another prominent DNA family to the picture of the large-scale organization of the sugar beet genome. Now that representatives of most of the major repetitive DNA families have been cloned it is feasible to investigate the fine structure of this chromosomal region by fluorescent in situ hybridization to pachytene chromosomes (Schmidt and Heslop-Harrison 1996) or perhaps extended chromatin fibres (Fransz et al. 1996).

The genomic organization in tandem repeats of the pSV family was studied by PCR with primers annealing to neighbouring repeats and by sequence analysis of pSV dimers, which also showed the head-to-tail orientation of repeats. In contrast to previously analyzed *Beta* satellite DNAs, repeats of the pSV satellite families are very homogeneous showing little divergence between repeats and conservation of restriction sites within the section *Beta* leading to the detection of mostly monomers after Southern hybridization (Fig. 2 A, B). Conserved higher-order units, detectable as strongly hybridizing fragments on Southern blots, may reflect an organization similar to the alphoid centromeric satellite DNA from humans (Willard and Waye 1987) although no chromosome-specific variants were observed. Within the seven pSV monomers analyzed there are only three single nucleotide deletions and only two variable GC-rich regions (Fig. 1A) were found, resulting in an overall divergence of less than 1%. This is considerably less heterogenity than other satellite families of sugar beet where sequence divergence ranges from 6 to 16%, and sometimes up to 23% in related wild beet species (Kubis et al. 1997). Differences in sequence homogenization between satellite families have been observed for two satellite DNA repeats of the genus *Cucurbita* (King et al. 1995). Homogenization mechanisms for members of tandem repeat families include unequal crossing over and gene conversion, although why these evolutionary processes act appearently only on a subset of all satellite DNA families within a plant genome remains to be clarified.

The dispersed sequence family pDRV, represented by three clones (Fig. 1 B), is more diverged, and highly amplified in the section *Beta*. The fluorescent in situ hybridization results (Fig. 4 D) revealed the interspersed nature of the pDRV sequence with local clustering in some interstitial regions. The strongly amplified fragment detectable in all species of the section *Beta* corresponds most likely to full-length repeating units which are cloned in pDRV1 and pDRV2, while the sequence in pDRV3 is a truncated variant.

A large proportion of dispersed repeats in plants are considered as derivatives or remnants of transposable elements. More than 13 complex families of dispersed repeats have been found in a 280 kb stretch of maize DNA (Bennetzen et al. 1994), and recent studies have shown that most of them belong to various classes of retroelements (SanMiguel et al. 1996). We could not identify any features in pDRV clones which resemble homology to retroelements or remnants thereof, although the chromosomal distribution of the pDRV family shows similarities to the physical distribution of retroelements in sugar beet (Schmidt et al. 1995; Kubis et al. 1998). Also, no similarity to tRNA-like sequences, as found in interspersed and tandem repeats of *Brassica* (Harrison and Heslop-Harrison 1995; Kapila et al. 1996), or to dispersed miniature-inverted repeat transposable elements (Bureau et al. 1994, 1996) could be detected. Therefore, the amplification and dispersion mechanism of the pDRV family is unknown.

Wild relatives of sugar beet beets have attracted the attention of breeders because of the resistance genes and agronomically desirable traits found in their gene pool (for a review see Van Geyt et al. 1990). These are valuable gene resources for beet breeding, and contain genes for cold or drought tolerance, disease resistance (rizomania, *Cercospora beticola*, nematodes, beet yellows virus disease), the monogermic seed character and apomixis. The differential amplification of the pDRV family in one of the four *Beta* sections may facilitate the labelling and detection of sugar beet chromosomes in interspecific hybrids by fluorescent in situ hybridization using the pDRV sequence as a probe. Moreover, the interspersed genomic organization of the pDRV sequences and their distribution over all 18 sugar beet chromosomes might, similar to the retrotransposon-based approach (Waugh et al. 1997), provide a starting point for the detection of

sequence-specific amplification polymorphisms (S-SAP) in *Beta vulgaris*.

Acknowledgements T. Schmidt acknowledges financial support by grants of the DFG (Schm1048/2-1 and Schm1048/2-2). We thank A. Schmidt for excellent technical assistance. Collaboration was facilitated by ARC grant 3131-ARC-XI-97/45 of the DAAD and the British Council.

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