Differential amplification of satellite PaB6 in chromosomally hypervariable
Prospero autumnale complex (Hyacinthaceae)

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- **Background and Aims** Chromosomal evolution, including numerical and structural changes, is a major force in plant diversification and speciation. This study addresses genomic changes associated with the extensive chromosomal variation of the Mediterranean *Prospero autumnale* complex (Hyacinthaceae), which includes four diploid cytotypes each with a unique combination of chromosome number \(x = 5\), \(6\), \(7\), rDNA loci and genome size.

- **Methods** A new satellite repeat \(PaB6\) has previously been identified, and monomers were reconstructed from next-generation sequencing (NGS) data of *P. autumnale* cytotype \(B'B'\) \((2n = 12)\). Monomers of all other *Prospero* cytotypes and species were sequenced to check for lineage-specific mutations. Copy number, restriction patterns and methylation levels of \(PaB6\) were analysed using Southern blotting. \(PaB6\) was localized on chromosomes using fluorescence *in situ* hybridization (FISH).

- **Key Results** The monomer of \(PaB6\) is 249 bp long, contains several intact and truncated vertebrate-type telomeric repeats and is highly methylated. \(PaB6\) is exceptional because of its high copy number and unprecedented variation among diploid cytotypes, ranging from \(10^1\) to \(10^6\) copies per \(1C\). \(PaB6\) is always located in pericentromeric regions of several to all chromosomes. Additionally, two lineages of cytotype \(B'B'\) \((x = 7)\), possessing either a single or duplicated SS rDNA locus, differ in \(PaB6\) copy number; the ancestral condition of a single locus is associated with higher \(PaB6\) copy numbers.

- **Conclusions** Although present in all *Prospero* species, \(PaB6\) has undergone differential amplification only in chromosomally variable *P. autumnale*, particularly in cytotypes \(B'B'\) and \(B'B'\). These arose via independent chromosomal fusions from \(x = 7\) to \(x = 6\) and 5, respectively, accompanied by genome size increases. The copy numbers of satellite DNA \(PaB6\) are among the highest in angiosperms, and changes of \(PaB6\) are exceptionally dynamic in this group of closely related cytotypes of a single species. The evolution of the \(PaB6\) copy numbers is discussed, and it is suggested that \(PaB6\) represents a recent and highly dynamic system originating from a small pool of ancestral repeats.

**Key words:** PaB6, Prospero autumnale, Hyacinthaceae, chromosomal evolution, copy number, differential amplification, fluorescence *in situ* hybridization (FISH), genome size, pericentric satellite DNA, next-generation sequencing.

INTRODUCTION

Genomes of higher plants contain a spectrum of repetitive DNAs (Schmidt and Heslop-Harrison, 1998; Macas et al., 2002; Ugarković and Plohl, 2002; Hemleben et al., 2007). This repetitive fraction is predominantly composed of dispersed mobile genetic elements (DNA transposons, retroelements) and tandemly repeated satellite DNAs (Hemleben et al., 2007; Weiss-Schneeweiss and Schneeweiss, 2013). Satellite DNA is typically species or genus specific, consisting of long arrays of late-replicating, tandemly arranged, head-to-tail repeats (Charlesworth et al., 1994; Richard et al., 2008).

Satellite DNA is a non-coding fraction of the genome of limited transcriptional capacity, subject to methylation, histone modification and chromatin remodelling (Volkov et al., 2006; Hemleben et al., 2007). It is preferentially localized in heterochromatic pericentromeric and sub-telomeric chromosomal regions, but also occurs interstitially (Charlesworth et al., 1994; Hemleben et al., 2007). No general function has been ascribed to satellite DNA (Ugarković and Plohl, 2002; Hemleben et al., 2007), although biological roles have been suggested for its specific families — the maintenance of chromosome structure (Ferree and Prasad, 2012), recognition of homologous chromosomes during meiosis (Willard, 1998; Ferree and Prasad, 2012), regulation of gene expression (Pezet et al., 2012), and heterochromatin organization and centromere function (Csink and Henikoff, 1998; Ugarković and Plohl, 2002; Ugarković, 2005; Hemleben et al., 2007; Martins et al., 2008; Gong et al., 2012; Pezet et al., 2012).

Higher plant genomes have from a few to many families of satellite DNAs (Hemleben et al., 2007; Macas et al., 2007, 2011). Individual satellite DNA families in a genome differ in sequence...
and copy number. Thus, one or a few families are usually present in high copy number, while others have low numbers of repeats (Hemleben et al., 2007). It has been proposed that groups of related taxa share a common ‘library’ of satellite DNA families, each of which may follow its own evolutionary trajectory (Meštrović et al., 1998). As species diverge, some satellite DNA families reduce in copy number, or even disappear, while others amplify, and new variants may arise (Meštrović et al., 1998; Nijman and Lenstra, 2001; Pons et al., 2004). Newly arising variants of a satellite DNA can rapidly replace previous copies due to concerted evolution, which results in intraspecific sequence homogenization (Plohl, 2010). The efficiency of homogenization is satellite DNA specific and depends on initial copy number, genomic location, repeat length and mode of reproduction (Dover, 1982; Stephan and Cho, 1994; Plohl et al., 2008; Navajas-Pérez et al., 2009; Kuhn et al., 2010). All these changes may parallel, or even precede, species diversification (Elder and Turner, 1995; Koukalova et al., 2010; Raskina et al., 2011; Belyayev and Raskina, 2013). Plant satellite DNA families are often derived from fragments of standard components of the genome, such as 3S rDNA (Lim et al., 2004; Almeida et al., 2012), 5S rDNA (Vittorazzi et al., 2011) or transposable elements (Sharma et al., 2013). Their subsequent evolution involves various processes such as replication slippage, unequal crossing-over, gene conversion or extrachromosomal circular DNA (eccDNA) formation (Smith, 1976; Walsh, 1987; Charlesworth et al., 1994; Elder and Turner, 1995; Cohen et al., 2008; Navrátilová et al., 2008).

The genus Prospero (Hyacinthaceae) consists of two chromosomally and morphologically stable species, P. hanburyi, 2n = 14 and P. obtusifolium, 2n = 8, and a chromosomally variable species complex referred to as P. autumnale. Prospero autumnale consists of a spectacular, and unparalleled, array of genetically, chromosomally and phylogenetically well-defined, recently evolved, diploid cytotypes, and a large array of polyploid derivatives (Vaughan et al., 1997; Jang et al., 2013). This complex shows near homogeneity in its morphology, and provides an excellent system for comparative and evolutionary genomic studies. It is distributed across the whole Mediterranean basin (Speta, 1998; Jang et al., 2013). Four chromosomally distinct diploid lineages (cytotypes) have been described, each of which possesses a unique combination of basic chromosome number (x = 5, 6, 7), DNA content and localization of rDNAs (Vaughan et al., 1997; Jang et al., 2013). Two cytotypes based on x = 7 are referred to as B^7B^7, distributed across the whole Mediterranean basin, and AA, which has larger chromosomes and genome size and is confined to the western-most Mediterranean and the Atlantic coast of Morocco, Portugal and Spain. The other two diploid cytotypes – with 2n = 12 (B^7B^5) and 2n = 10 (B^5B^5) – originated from a putative ancestor with 2n = 14 via independent chromosome fusions. B^7B^7 is endemic to Crete while B^5B^5 is endemic to Libya. With the exception of the most recently evolved cytotype B^5B^5, all diploids hybridize and undergo polyploidization in nature to give auto- and allopolyploids. Amongst polyploids, tetraploid and hexaploid cytotypes are most common and widespread (Ainsworth et al., 1983; Vaughan et al., 1997).

Phylogenetic and evolutionary relationships of the three species of Prospero have recently been established, and the ancestral basic number for the P. autumnale complex was inferred to be x = 7 (Jang et al., 2013). Evolution of the cytotypes AA and B^7B^5 has been shown to be accompanied by independent genome size increases (Jang et al., 2013). Large heterochromatic blocks, however, have been detected only in cytotype B^5B^5 (Ebert et al., 1996).

Recent developments in high-throughput next-generation sequencing (NGS; Margulies et al., 2005) allow in-depth analyses of all components of any genome (Wicker et al., 2009; Deschamps and Campbell, 2010), and thus rapid identification of satellite DNAs (Macas et al., 2007; Torres et al., 2011; Heckmann et al., 2013). The current study involves comparative evolutionary analysis of a satellite PaB6 identified by NGS from cytotype B^5B^5. Specifically, the aims are to: (1) isolate, characterize, and determine the abundance and localization of PaB6 in the diploid species and cytotypes of Prospero, and their homopoloid diploid hybrids; (2) assess intra- and interspecific variation of the reconstructed PaB6 monomer at all levels of its organization – its DNA sequence, chromosomal localization and genomic abundance; (3) analyse, in a phylogenetic context, the evolutionary trajectories of PaB6 in all six diploid cytotypes of P. autumnale and their diploid homopoloid hybrids; and (4) discuss the dynamics of PaB6 evolution in the context of major chromosomal rearrangements in the genus.

MATERIALS AND METHODS

Plant material and DNA isolation

Plants from collections of F. Speta, Linz, and J. S. Parker, Cambridge, were grown in the Botanical Garden of the University of Vienna. The plants studied and their collection details are listed in Supplementary Data Table S1. Due to the high levels of chromosomal variation in Prospero (Jang et al., 2013), every plant was karyotyped prior to analysis. Only ‘standard’ individuals without structural chromosomal variants were used.

Total genomic DNA was isolated from leaves, of several individuals each, of P. obtusifolium, P. hanburyi and the four diploid cytotypes of P. autumnale, including homopoloid diploid hybrids (Supplementary Data Table S1) using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987; Jang et al., 2013).

Next-generation sequencing and clustering-based repeat identification

Sequencing of randomly sheared total genomic DNA of the cytotype B^5B^5 of P. autumnale was performed by the Center for Medical Research, Graz, Austria using a Roche/454 GS FLX instrument with Titanium reagents (Roche Diagnostics). Sequencing half a 70 × 75 picotitre plate yielded 555 480 reads of average length 350 bp. Quality-filtered reads (397 694 corresponding to 2.2 % coverage of the genome) were subjected to graph-based clustering analysis, as described by Novák et al. (2010), to identify groups of reads representing repetitive elements (H. Weiss-Schnieweiss et al., unpubl. res.). One hundred and ninety-five out of a total of 19 751 clusters, corresponding to the most abundant families of genomic repeats, were analysed for their similarity to known sequences using RepeatMasker Open-3.0 (http://www.repeatmasker.org) and BLAST (Altschul et al., 1990) searches against GenBank.

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databases and a database of plant mobile element protein sequences (Novák et al., 2013). Graphical layouts of individual clusters were examined using the SeqGrapheR program (Novák et al., 2010).

Characterization of monomers of satellite repeats

Only one genomically abundant cluster (CL0009) was identified amongst all clusters as containing a potential satellite repeat. Structural features of the tandem repeat motif and its sub-repeats within the contigs of this cluster were further analysed with DOTTER (Sonnhammer and Durbin, 1995). Identification of the most conserved sequence variants and consensus monomer reconstruction of satellite repeat PaB6 were conducted using k-mer frequency analysis as described previously (Macas et al., 2010), using 25 bp long k-mers for final sequence reconstruction.

PCR amplification, cloning, sequencing and phylogenetic analysis of PaB6

The reconstructed consensus sequence of the monomer of PaB6 was used for the design of oligonucleotide primers (PaB6F, 5'-ACCCTAATCGAAGCTGCT; PaB6R, 5'-TAGAGTTATGGGATGTGTAAC) facing outwards (Fig. 1A). These primers were used for amplification of PaB6 monomers from genomic DNA of diploid Prospero species and cytotypes and three outgroup species (all of family Hyacinthaceae; Supplementary Data Table S1). Polymerase chain reactions consisted of 1 × buffer (MBI Fermentas, St Leon-Rot, Germany), 2.5 mM MgCl2 (MBI Fermentas) 0.5 mM of each of the dNTPs (MBI Fermentas), 0.2 μM of each primer (Sigma Aldrich, Vienna, Austria) and 1 U of RedTaq polymerase (Sigma Aldrich). Amplification was performed on an ABI thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA) with the initial 3 min at 94 °C followed by 25 cycles each of 45 s at 94 °C, 45 s at 55 °C and 40 s at 72 °C, and a final elongation step at 72 °C for 10 min. Amplified fragments were separated on a 1.5 % agarose gel, and PCR products corresponding to the length of the monomers of satellite DNA PaB6 were purified from the gel using Invisorb® Fragment clean up (Invitek, Berlin, Germany). DNA was cloned using the pGEM-T Easy vector system and JM109 competent cells (Promega, Madison, WI, USA) following the manufacturer’s instructions. Five inserts per individual were amplified from plasmids using colony PCR with universal M13 primers whereby recombinant colonies were added directly into the PCR mix and inserts amplified using reagents and conditions described in Park et al. (2007). Amplification products were treated with exonuclease I (ExoI) and calf intestine alkaline phosphatase (CIAP) according to the manufacturer’s protocol (MBI Fermentas), and amplicons were cycle sequenced using Big Dye terminator chemistry (Applied Biosystems) and run on a 48 capillary ABI 3730 DNA Analyzer (Applied Biosystems). The sequences of satellite DNA were manually aligned in BioEdit v.7.0.9 (Hall, 1999). Phylogenetic analyses were performed using Splitstree (version 4.11.3; Huson and Bryant, 2006). Sequenced clones are available from GenBank under accession nos KF897587–KF897652 (Supplementary Data Table S1). Gradient PCR was performed on a peqstar thermocycler (peqlab, Erlangen, Germany) to check for the presence of PaB6 in related genera. Primers, the PCR set-up and the PCR program used were the same as described above, except that the annealing temperatures ranged from 50 to 55 °C (Fig. 2, and not shown).

Southern and slot blot hybridization

Abundance and restriction patterns of PaB6 monomers in selected individuals were analysed using the Southern blot technique. A 1 μg aliquot of total genomic DNA of each Prospero species and cytotype was digested with 0.7 μL of BstNI restriction endonuclease for 2 h at 37 °C. Digested DNA fragments were separated on a 1 % (w/v) agarose gel and transferred onto a positively charged nylon membrane, Hybond-XL, by the capillary flow method.

The probe used for hybridization was a 249 bp PCR product representing the PaB6 satellite of P. autumnale cytotype B#6 (clone 4 of individual H195; GenBank accession no. KF897620). The probe was labelled either radioactively with 32P (DekaLabel kit, MBI Fermentas, Vilnius, Lithuania) or using a DIG-nick translation kit (Roche, Vienna, Austria). Radioactively labelled probe was hybridized to the membrane and washed under high-stringency conditions, as described in Matýšek et al. (2011). Hybridization bands were visualized with a PhosphorImager (Storm, Molecular Dynamics, Sunnyvale, CA, USA), and the data were processed in ImageQuant software (Molecular Dynamics).

Hybridization of digoxigenin-labelled probe (Dig Easy Hyb, Roche, Germany) to genomic DNA was carried out at 43 °C for 14 h, and it was then washed twice in 2 × SSC (saline-sodium citrate buffer) containing 0.1 % SDS (sodium dodecylsulphate) for 5 min at room temperature, and twice in 0.5 × SSC containing 0.1 % SDS for 15 min at 65 °C. Probe was detected with CSPD chemiluminescent substrate (Roche Applied Science, USA) using Dig Wash and Block Buffer Set (Roche Applied Science, Germany), and the hybridization signals were visualized on Fusion FX7 Advance (peqlab). Due to the lower sensitivity of chemiluminescent detection compared with radioactive systems, an additional hybridization experiment was performed with cytotypes B#7, which had been shown to possess lower amounts of satellite DNA, using 1 μg and additionally also 3 μg of genomic DNA.

The copy number of PaB6 in all species and cytotypes was estimated using the slot blot technique. Briefly, the DNA concentration was estimated using Nanodrop 3300 (peqlab) with PicoGreen (Invitrogen) as DNA stain. Two or three dilutions of genomic DNA (100, 20 and 2 ng for B#B and B#B cytotypes; 2000, 200 and 20 ng for B#B and AA cytotypes; 2000 and 200 ng for P. hanburyi and P. obtusifolium), together with a series of dilutions of the unlabelled PaB6 insert corresponding to the monomer sequence, were denatured in 0.4 M NaOH and neutralized with 0.75 M NH4OAc. Samples were blotted onto a positively charged Nylon membrane (peqlab) using a vacuum slot blotter (VWR, Vienna, Austria). The probe and the hybridization conditions used were the same as described above for non-radioactive Southern hybridization. Copy number was estimated using Fusion FX7 Advance software (peqlab).

Methylation levels

The methylation level of PaB6 repeats in the B# genome was assessed using a radioactive Southern blot (see above). The
**FIG. 1.** *PaB6* monomer characterization. (A) Monomer sequence logo (Schneider and Stephens, 1990) with the height of the letters corresponding to $k$-mer frequencies. Arrows indicate the origin and direction of forward and reverse primers (underlined). Perfect telomeric sequences are underlined in red, and imperfect variants in violet. (B, C) Dot plots of the monomer sequence against itself with lower (B) and higher similarity stringency (C).
genomic DNA was digested with two restriction enzymes – BsrNI (CCWGG) and ScrFI (CCNGG) – which recognize and cut nearly the same sequence, with ScrFI being sensitive to the inner C methylation.

Fluorescence in situ hybridization

Chromosomes were prepared by enzymatic digestion and squashing (Jang et al., 2013). Fluorescence in situ hybridization (FISH), probe labelling and detection were carried out according to the method of Jang et al. (2013).

The probes used for FISH were a monomer of satellite DNA PaB6 from the B7 genome in plasmid pGEM-T Easy and the genomic region of 5S rDNA from P. hanburyi (H397, H115); 11–12, P. obtusifolium (H559; H563; Supplementary Data Table S1)). (B) Gradient PCR amplification of PaB6 monomers in selected Prospero samples and outgroup taxa [M, marker; 1, P. obtusifolium H559; 2, P. autumnale B'B'; 3–4, B'B' (H424, H428); 5–6, B'B' (H582, H640); 7–8, AA (H541, H550); 9–10, P. hanburyi (H397, H115); 11–12, P. obtusifolium (H559; H563; Supplementary Data Table S1)]. (C) Neighbour-net of PaB6 repeats cloned from diploid cytotypes of P. autumnale (AA, open circles; B'B', black filled squares; B'B', black filled triangles; B'B', black crosses), P. obtusifolium (grey circles) and P. hanburyi (grey triangles).

RESULTS

Satellite DNA identification and characterization of the monomers

Clustering analysis of the shotgun Roche/454 reads of Prospero autumnale cytotype B'B' (2n = 12) produced thousands of clusters differing in size, corresponding to the sequence composition and genomic abundance of the various genomic repeats. A set of 195 of the largest clusters, representing the most abundant repetitive elements with genome proportions exceeding 0.01 %, was searched for features typical of satellite repeats. Only one such cluster was identified, based on the shape of the cluster graph (Novák et al., 2010) and the presence of tandem repeats in assembled contigs (Fig. 1; Supplementary Data Fig. S1). This novel satellite has been designated as PaB6 – satellite DNA isolated from P. autumnale (Pa) cytotype B'B' (B6). The number of reads in the cluster was 8461, or 1.8 % of the total, giving an estimate of the proportion of PaB6 in the genome. The consensus sequence reconstruction using 25 bp long k-mers (Macas et al., 2010) resulted in a monomer of 249 bp in length (Fig. 1A), with a GC content of 44 %. Detailed analysis, using the NGS dataset, revealed two large truncated sub-repeats which could have given rise to the present-day higher order monomer of 249 bp (Fig. 1B). Each of the two sub-repeats is typically composed of three even smaller secondary sub-repeats (Fig. 1C).

The complex structure of this monomer is also indicated by the pattern of PaB6 amplification using PCR (see below).

The monomer of PaB6 contains seven intact vertebrate-type telomeric repeats (TTAGGG) dispersed amongst other sequences and in two instances forming dimers (Fig. 1A).
Additionally, five imperfect telomeric-like repeats have been identified, and potentially other repeats degenerated to a higher degree (Fig. 1A). A penta-nucleotide CAAAA, conserved in many satellites (Macas et al., 2002), occurred three times on the top strand. In addition, there were four A4 tracts important for DNA conformation and chromatin folding (Plohl et al., 2010).

### Comparative sequence analysis of the monomers

The PCR amplification of the major type of the monomer, using primers designed for the reconstructed B6 genome monomer, resulted in products of the expected length in all four diploid cytotypes of *P. autumnale* and the two related species, *P. hanburyi* and *P. obtusifolium*. PCR with PaB6-specific primers yielded one strong band of approx. 250 bp, corresponding and *P. hanburyi* resulted in products of the expected length in all four diploid known quantities of genomic DNAs of all three species and PaB6 cent dot blot hybridization of labelled *PaB6* primers designed for the reconstructed B6 genome monomer, the monomer of *P. autumnale* ing temperature (Fig. 2B). Very faint, monomer-related bands, observed, or observed very rarely. Amplification of dimers or even longer fragments was not from two or three individuals of each of the six taxa/cytotypes. The outgroup taxa of the family Hyacinthaceae were subjected to the same PCR amplification protocol and primers. Representatives of the related genera *Othocallis* and *Barnardia* showed no bands after PCR amplification, regardless of the annealing temperature (Fig. 2B). Very faint, monomer-related bands, close to the limit of detection, were seen occasionally, without any consistent pattern regarding annealing temperature or taxon, and were regarded as contamination (Fig. 2B, and not shown).

Sequence analysis of 66 cloned *PaB6* monomers (Supplementary Data Table S1), representing monomers amplified from two or three individuals of each of the six taxa/cytotypes, confirmed that they all carried *PaB6* repeats. Fifty-one of these (83 %) were (93–100 %) and between (92–100 %) the different diploid cytotypes of *P. autumnale* and two other *Prospero* species, were observed (Supplementary Data Table S2). Thus, the intercytotype sequence variation of repeats amplified with the reconstructed monomer primers was as equally low and random as that within cytotypes or between individuals. The variation was mostly due to single base pair indels or point mutations occurring at different positions along the monomer, and these were monomer specific (alignment available upon request).

Neighbour-net analyses of DNA sequences of all cloned inserts of *PaB6* repeats from the six cytotypes corroborated the analyses of variation within the monomers, and did not reveal any cytotype-specific lineages (Fig. 2C). Instead, the repeats originating from different individuals were intermingled, regardless either of *PaB6* overall copy number and abundance or of their phylogenetic relationship.

### Chromosomal localization and organization of *PaB6* repeats

*PaB6* has been localized in all six cytotypes using FISH (Supplementary Data Table S3). The variation in number and size of satellite DNA loci detected corresponded well to the Southern slot results. Thus *P. obtusifolium* (Fig. 4A) and *P. hanburyi* (Fig. 4B) had no *PaB6* loci detectable by FISH due to very low copy numbers of *PaB6* monomers (Figs 3A and 4A–B). *Prospero autumnale* diploids, in contrast, all exhibited hybridization signals using FISH, but were variable in numbers of sites and in signal strengths (Fig. 4C–K). *PaB6* is predominantly located in pericentromeric regions of at least one, and sometimes all, chromosome pairs, and might, at least partly, span the centromeres (Supplementary Data Fig. S2).

In cytotype B5B5, major loci were present on all chromosomes of the complement (Fig. 4F, G). The pattern of satellite distribution was remarkably uniform between individuals and populations, and loci were of similar signal strength. Chromosome 1 showed the only polymorphism, with the locus size varying between homologues in some individuals (Fig. 4F, G).

In B5B5, *PaB6* loci occurred on four of the five chromosome pairs (Fig. 4H) and were of similar signal strength.
Chromosome 3 showed, at most, a very weak hybridization (H637, H565); (D) B7B7 (H424, duplicated 5S1 rDNA) and B7B7 (H428, single 5S1 rDNA) each with 1 and 3 μg of DNA.

Chromosome 3 showed, at most, a very weak hybridization signal (Supplementary Data Table S3, and data not shown).

Cytotype AA had only a single locus of PaB6 – on chromosome 5 – but this was weak and barely detectable (Fig. 4C). The most variable PaB6 distribution was shown by cytotype B7B7. Some individuals possessed medium-sized signals in pericentromeric regions of all chromosomes (Fig. 4E; Supplementary Data Table S3), while others had much weaker signals limited to three chromosome pairs (Fig. 4D; Supplementary Data Table S3). These patterns correlated with a duplication polymorphism of 5S rDNA present on chromosome 1 (5S1; see also Fig. 6). Thus the five individuals with a single 5S1 rDNA locus showed moderate amplification of Path on all chromosomes (Fig. 4E), while the six plants with a duplicated 5S locus carried weakly amplified Path loci only on chromosomes 1, 2 and 4 (Fig. 4D). The number and localization of Path satellite DNA loci in all cytotypes are shown in Fig. 6 and Supplementary Data Table S3.

All six F1 diploid hybrids possessed perfectly additive numbers and strengths of Path loci compared with their diploid parents. This was supported by Southern blot hybridization of a B7B7 hybrid, which also indicated additivity (not shown).

The Path monomer contains seven perfect and a few imperfect vertebrate-type telomeric sequences (TTAGGG), typical of the monocot order Asparagales to which Prospero belongs. TTAGGG sequences were detected at chromosome ends (Fig. 5) but additionally co-localized with the Path loci. Signal intensity in the pericentric chromosome regions using a telomeric DNA probe corresponded to signal strength and localization of the Path probe itself (Fig. 5A, B).

DISCUSSION

Tandem repeats localize to heterochromatic segments in chromosomes (Hemleben et al., 2007). Prospero cytotypes differ in the amount and distribution of heterochromatin, both among and within cytotypes. So far, only the cytotypes AA, B7B7 and B7B7 have been analysed using C-banding (Ebert et al., 1996) and the only consistently detectable heterochromatic blocks co-localized with nucleolar organizer regions (NORs). However, cytotype B7B7 had a high amount of heterochromatin, detected as blocks (C-bands) in the pericentric regions of all chromosomes (Ebert et al., 1996). This was the rationale for selecting the B7 genome for repetitive DNA fraction analyses. Cytotype B7B7 was very variable in the number of heterochromatic blocks, but these were mainly dot-like and localized interstitially, except for slightly larger pericentric blocks which varied in size between individuals. Cytotype AA had only small interstitial heterochromatic blocks on six of the seven pairs. All of these pericentric heterochromatic blocks detected by Ebert et al. (1996) correspond to Path signals. The additional, smaller and more polymorphic interstitial bands detected are most likely to be composed of other tandem repeat(s), some of which might be cytotype specific.

Satellite DNA repeats represent a substantial proportion of the genomes of many higher plants (e.g. VicTR-A/B in Vicia, Macas et al., 2000; FriSAT1 in Fritillaria, Ambrozová et al., 2011). The PaB6 repeat of Prospero is one of the most abundant satellites reported so far (Hemleben et al., 2007). It represents about 10% of the genome in the B7B7 cytotype with 1 × 10^6 copies. In comparison, tandem repeat VicTR-A/B comprises about 1% of the genome of most Vicia species with 10^6 copies (VicTR-A) but reaches 25% of the genome with 1 × 10^9–5 × 10^9 copies (VicTR-B) in V. sativa (Macas et al., 2000), approaching the highest value reported in plants for the
FIG. 4. Localization of PaB6 in chromosomes of diploid Prospero species and cytotypes, and in three homoploid hybrids. The PaB6 loci are shown as green signals, and 5S rDNA in red. (A) P. obtusifolium (H563), (B) P. hanburyi (H115), (C–K) P. autumnale complex: (C) cytotype AA (H551, inset: chromosomes carrying PaB6 signals), (D, E) B7B7 with duplicated (D: H424, left inset, duplicated 5S rDNA signals; right inset, chromosomes carrying PaB6 signals) and single (E: H440) 5S rDNA locus in chromosome 1, (F, G) B6B6 with weak (F: H195) and strong (G: H427) signal of PaB6 in chromosome 2 (arrows), (H) B5B5 (H581), (I) AB5 (H567), (J) B5B7 (H633), (K) B6B7 (H518) diploid hybrid. Each individual has a unique ID (in parentheses, e.g. H563; see Supplementary Data Table S1). Scale bar = 5 μm.

TABLE 1. Characterization of satellite PaB6 repeats in diploid species and cytotypes of the genus Prospero

| Taxon          | 2n | Genome proportion % of PaB6 | Copy number/1C of PaB6 | Genome size (pg) per 1C* | Figure |
|---------------|----|-----------------------------|------------------------|--------------------------|--------|
| P. autumnale  |    |                             |                        |                          |        |
| Cytotype B5B5 | 10 | 6.3–7.4                     | 1.25–1.37 × 10⁶        | 4.86 ± 0.002             | 2A; 3A; C; 4H |
| Cytotype B5B5 | 12 | 7.16–10.71                  | 1.76–2.06 × 10⁶        | 6.27 ± 0.083             | 2A; 3A–C; 4F; G; 5A, B |
| Cytotype B5B5: single 5S rDNA | 14 | NA                          | NA                     | 4.23 ± 0.048             | 2A; 3B; D; 4E |
| Cytotype B5B5: duplicated 5S rDNA | 14 | 0.12–0.14                  | 2.11–2.49 × 10⁴       | 4.45 ± 0.023             | 2A; 3A, D; 4D |
| Cytotype AA   | 14 | 0.06–0.08                   | 1.75–2.56 × 10⁴       | 7.85 ± 0.045             | 2A; 3A; 4C |
| P. hanburyi   | 14 | ND                          | ND                     | 6.81 ± 0.017             | 2A; 3A; 4B |
| P. obtusifolium | 8 | ND                          | ND                     | 4.94 ± 0.039             | 2A; 3A; 4A |

NA, not analysed due to lack of material; ND, copy number could not be determined due to very low PaB6 contents.

*Jang et al. (2013).
FokI element in V. faba (2.5 × 10^7 copies/1C; Kato et al., 1984). The 37–55 bp long PAF1 repeat in Picea abies occurs in 2.7 × 10^6 copies/1C (approx. 0.6%; Sarri et al., 2008), while MCSAT in M. comosum has 9 × 10^5 copies representing 5% of the genome (de la Herrán et al., 2001).

PaB6 is exceptional for its copy number variation between the closely related diploid cytotypes of one species complex. The satellite can clearly expand from a few hundred base pairs up to several hundred megabases in a relatively short evolutionary period. Such rapid changes should be reflected by genome size differences between Prospero cytotypes. The genome sizes of the derived cytotypes B^5B^5 and B^6B^6 are distinctly higher than those of cytotype B^3B^3, which has been inferred to be most similar to the ancestral karyotype (Jang et al., 2013; K. Emadzade et al., unpubl. res.). PaB6 amplification significantly contributes to these genome size increases and gives rise to heterochromatic blocks in B^6B^6. The correlation between genome size and PaB6 amount is particularly evident in the comparison of the youngest cytotype B^3B^3 and its close relative, and likely ancestor, B^3B^3′ (Jang et al., 2013). The B^3 genome is about 400 Mb (10%) larger than the B^3′ genome, half of which can be attributed to PaB6 copy number increase (325 Mb in B^6B^6 vs. 7 Mb in B^3B^3′). In contrast, the large size of the A genome is clearly not associated with the high copy number of PaB6.

Satellite DNA copy number can change relatively rapidly due to expansions and contractions of satellite arrays. Thus, the copy number of FRISAT1 in the genus Fritillaria varies within and between different subgenera (Ambrožová et al., 2011), and several genus-specific satellite DNAs differ in copy numbers between related Secale (Cuadrado and Jouve, 2002) and Nicotiana species (Lim et al., 2004). Such differences are also observed between varieties and cultivars of Phaseolus vulgaris and maize (Peacock et al., 1981; Ribeiro et al., 2011) indicating the highly dynamic character of satellite repeats. These changes may be accompanied by divergence of the monomer sequences during evolution, via accumulation and fixation of mutations in satellite families (Plohl et al., 2008). Interestingly, in Prospero, despite the dynamic changes in copy number, there is no indication of sequence divergence during lineage evolution.

In Barnardia and Othocallis (Fig. 2B), genera closely related to Prospero (Pfösser and Speta, 1999; Ali et al., 2012), no PaB6 monomers were detected, shown by a lack of amplification of PaB6 monomer-equivalent bands in PCR. Thus, PaB6 probably evolved during the emergence of the genus Prospero, and remained in low copy number as part of the library of repeats (Meštrović et al., 1998) in the chromosomally stable species P. obtusifolium and P. hanburyi. PaB6 amplification, therefore, is specific to the chromosomally dynamic P. autumnale complex.

PaB6 dynamics can be assessed against the phylogeny of the genus (Jang et al., 2013). Prospero obtusifolium and P. hanburyi possess very few PaB6 monomers, and these can only be detected by PCR, because they are below the detection limit of all types of in situ hybridization. In contrast, the four diploid cytotypes of P. autumnale all possess PaB6 in amounts detectable by FISH and genomic DNA hybridization, although copy number varies substantially. PaB6 in B^6B^6 represents 8–10% of the genome and 6–7% in B^3B^3. Copy number estimation from NGS data, however, suggests that PaB6 represents about 1.8% of the B^3′ genome, only a quarter of that from slot blot hybridization. This discrepancy is probably caused by PaB6 under-representation due to a bias affecting template preparation from satellite repeats during 454 sequencing (Macas et al., 2007; J. Macas et al., unpubl. res.).

In some plant and animal groups, patterns of copy number variation of a satellite DNA family in a group of closely related taxa carry a phylogenetic signal. However, similarity in copy number might result from independent satellite amplifications or contractions (Rosato et al., 2012). The two Prospero cytotypes whose genomes are enriched in PaB6 have reduced basic chromosome numbers of x = 6 and x = 5 derived from x = 7 via independent fusion events, so do not demonstrate a sister relationship (Jang et al., 2013; Fig. 6). Thus, the raised amounts of PaB6 in these two cytotypes could have resulted from independent amplifications, coinciding with fusions leading to basic number changes. This is particularly plausible for the phylogenetically young cytotype B^3B^3, which is nested within B^6B^6, a cytotype carrying relatively few copies of PaB6 (Jang et al., 2013; Fig. 6). However, high copy numbers in these two unrelated lineages might be a remnant of a common amplification event which was followed by differential loss. This hypothesis is more plausible for cytotype B^6B^6 than for B^3B^3. B^6B^6
clearly originated from \( x = 7 \), but does not strongly relate, phylogenetically or chromosomally, to any lineage of present-day \( B' B'' \), and may have arisen directly from the ancestral cytotype, or an as yet undiscovered \( B'' \) lineage, with high copy numbers of \( PaB6 \) (Jang et al., 2013). Thus, the lack of phylogenetic evidence of copy number of \( PaB6 \) in the ancestral karyotype of \( Prospero \) leaves the question open.

The presence of telomeric motifs in the \( PaB6 \) sequence is interesting with respect to the high karyotype instability within and between all \( P. autumnale \) cytotypes (Vaughan et al., 1997; Jang et al., 2013). The presence of interstitial telomeric repeats (ITRs) is often interpreted as a remnant of evolution by telomere–telomere chromosomal fusions. However, it may also result from rearrangements such as translocations or inversions (Uchida et al., 2002; Ruiz-Herrera et al., 2008; Rosato et al., 2012), particularly whole chromosomal arm inversions involving both the centromere and telomere (Presting et al., 1996). The occurrence of telomeric repeats within, or at the margins of, constitutive heterochromatin has been reported in vertebrates (Meyne et al., 1990) but is also known in plants (Presting et al., 1996; Uchida et al., 2002; Weiss-Schneeweiss et al., 2004; Minarec et al., 2009; Gong et al., 2012; He et al., 2013). It has been argued that these telomeric repeats can be an integral and long-established part of the satellite DNAs of constitutive heterochromatin (Slijepcevic et al., 1996; Garrido-Ramos et al., 1998; Metcalfe et al., 2004), originally inserted and amplified through DNA double strand breaks (DSBs) repaired by telomerase (Nergadze et al., 2004, 2007). The ITRs detected in \( Prospero \) are certainly an integral part of \( PaB6 \) interspersed amongst other sequence motifs. Their origin, however, cannot be unambiguously established.

Two mechanisms have been proposed for satellite DNA copy number change: unequal crossing-over with gene conversion (Liao, 1999; Eickbush and Eickbush, 2007), and amplification and homogenization of monomers by extrachromosomal circular DNA (eccDNA, ‘rolling circle’) molecules during recombination (Navrátilová et al., 2008; Cohen et al., 2010). They are not mutually exclusive and might operate in concert, resulting

\[ \text{FIG. 6. Model of evolution of PaB6 in diploid taxa of Prospero. Idiograms of all analysed species and cytotypes are mapped onto the ITS (internal transcribed spacer) tree (adapted from Jang et al., 2013). PaB6 satellite DNA is indicated as blue blocks, 5S rDNA as red circles and 35S rDNA as green circles. Asterisks indicate lineages which have experienced significant amplification of PaB6. Arrows mark amplification events accompanying fusions.} \]
in mobility and homogenization of repetitive DNAs. Whether these mechanisms are also involved in expansion of PaB6 in Prospero remains unknown.

Although copy number varies hugely between cytotypes within Prospero, the monomer sequence is conserved. This may indicate either relatively recent amplification of the monomer or efficient systems of sequence homogenization and gene flow between taxa (Hemleben et al., 2007). The geographically disjunct distributions of the cytotypes AA, BB’ and BB”, and consequent lack of gene flow between them, suggest that PaB6 represents a recent and highly dynamic system originating from a small pool of ancestral repeats (Mraivac et al., 2005; Plohl et al., 2010).

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SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: NGS graph layouts and dot-plots of the contigs. Figure S2: pericentric localization of PaB6 in cytotype BB’. Figure S3: methylation levels of PaB6. Table S1: plants used and collection details, GenBank accession numbers (PaB6), and methods used for analysis. Table S2: sequence similarity of cloned monomers of Prospero. Table S3: characterization of SS rDNA and satellite DNA PaB6 loci in chromosomes of diploid species, cytotypes and hybrids of the genus Prospero.

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