Structural polymorphisms and distinct genomic composition suggest recurrent origin and ongoing evolution of B chromosomes in the *Prospero autumnale* complex (Hyacinthaceae)

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**Summary**

- Supernumerary B chromosomes (Bs) are genomic parasitic components, originating from the A complement via chromosomal rearrangements, which follow their own evolutionary trajectories. They often contain repetitive DNAs, some shared with regular chromosomes and some newly evolved. Genomic composition, origin and evolution of Bs have been analysed in the chromosomally variable *Prospero autumnale* complex.
- Two rDNAs and a satellite DNA (PaB6) from regular chromosomes were mapped to Bs of 26 plants from three diploid cytotypes, their hybrids and polyploid derivatives. In homoploid diploid hybrids, genomic *in situ* hybridization (GISH) allowed B painting with the parental DNAs.
- Bs were structurally variable and highly enriched in 5S rDNA and satDNA PaB6, and rarely in 35S rDNA. Eleven combinations of rDNA and PaB6 localization were observed. The quantities of PaB6 in Bs and regular chromosomes were not correlated, suggesting amplification mechanisms other than recombination. PaB6 and 5S rDNA amounts increased with increasing ploidy level. GISH revealed two independent origins of Bs.
- The structural variation, repeat content, repeat-type fluctuations and differing genomic affinities of Bs in different cytotypes suggest that they represent young proto-B chromosomes. Bs in *P. autumnale* probably form recurrently as by-products of the extensive genome restructuring within this chromosomally variable species complex.

**Introduction**

Karyotypes of many species contain supernumerary genetic material, either as free B chromosomes (Bs) or inserted on standard chromosomes as supernumerary chromosomal segments (SCSs). SCSs are more frequent in insects than in plants, but are most notable in the monocotyledonous family Hyacinthaceae (Greilhuber & Speta, 1978; Ruiz Rejón & Oliver, 1981; Jamilena *et al.*, 1995; Ebert *et al.*, 1996; Garrido-Ramos *et al.*, 1998; Weiss-Schneeweiss *et al.*, 2004). B chromosomes, by contrast, have been reported in numerous species of animals, fungi and flowering plants (Jones, 1995; Camacho *et al.*, 2000). In plants, they are more common in monocots than in dicots, with hot spots in Liliales and Commelinaceae (Levin *et al.*, 2005). B frequencies in diploids and polyploids are similar (Jones & Rees, 1982; Trivers *et al.*, 2004), but are higher in families with large genome sizes (Trivers *et al.*, 2004; Levin *et al.*, 2005; Jones *et al.*, 2008).

Bs do not recombine with the A complement and so are exempted from strictly Mendelian inheritance and follow their own evolutionary trajectories (Camacho *et al.*, 2000; Jones *et al.*, 2008; Houben *et al.*, 2013). Bs are most frequently found in low numbers (0–5), but as many as 34 have been reported in an individual of *Zea mays* (Jones & Rees, 1982; Jones *et al.*, 2008). They are usually smaller than the standard complement and vary in size from dot-like micro-Bs (Houben *et al.*, 1997, 2013; Jones *et al.*, 2008) to chromosomes as large as the smallest chromosomes of the regular set (Jones *et al.*, 2008). Their size and structure are often stable within taxa (*Secale cereale* Marques *et al.*, 2012, 2013), but plants carrying more than one structural B type are well known (Guillén & Ruiz Rejón, 1984; Parker *et al.*, 1991).

The occurrence of Bs in phylogenetically unrelated groups indicates their independent and multiple origins (Levin *et al.*, 2005). Several hypotheses have been proposed to explain the origins of Bs from A chromosomes (Levin *et al.*, 2005; Sharbel *et al.*, 2005; Jones *et al.*, 2008; Martíx *et al.*, 2012; Houben *et al.*, 2013; Weiss-Schneeweiss & Schneeweiss, 2013), with most favouring their origin as a by-product of chromosomal rearrangements of the regular (A) set of chromosomes stimulated by hybridization.
or polyploidization (Jones & Houben, 2003; Houben et al., 2013). Recent support for this hypothesis has come from the genera Plantago (Dhar et al., 2002) and, in particular, Secale (Martis et al., 2012). Newly arisen chromosomal fragments often accumulate sufficient differences in structure and/or chromatin composition to ensure their meiotic isolation from A chromosomes, and establish meiotic and mitotic drive mechanisms to secure their own transmission to the next host generation (Langdon et al., 2000; Marschner et al., 2007; Jones et al., 2008; Banaei-Moghaddam et al., 2012; Klemme et al., 2013).

During their evolution, Bs capture coding and noncoding DNA sequences from A chromosomes (Maluszynska & Schweizer, 1989; Dhar et al., 2002; Kubalaková et al., 2003; Carchillan et al., 2009; Banaei-Moghaddam et al., 2012; Marques et al., 2012) and from organelar DNAs (Martis et al., 2012; Ruban et al., 2014), but novel B-specific repeats also evolve (Langdon et al., 2000; Martis et al., 2012; Klemme et al., 2013). Despite their abundance, the roles of Bs remain enigmatic, although many different effects on the carrier organism have been demonstrated, including influences on A-chromosome meiotic pairing (Jones et al., 2008; Houben et al., 2013).

An attractive system in which to establish patterns of chromosome evolution is the genus Prospero (Hyacintaceae). P. autumnale, one of three species of this genus, is itself a complex which includes four evolutionarily well-established diploid cytotypes (AA, B7B7, B6B6, B7B2; Jang et al., 2013). Each cytotype is characterized by a unique combination of basic chromosome number, genome size and pattern of rDNA and satellite DNA PaB6 distribution (Jang et al., 2013; Emadzade et al., 2014).

Polyploidy is frequent in the complex, resulting in autoploids of genome B7 (x = 7), most commonly 4x and 6x; but up to 20x (Ainsworth, 1981; Ebert, 1993; Speta, 1993, 2000), and two classes of allopolyploids – of A (x = 7) and B7 origin, and of B6 (x = 6) and B7 origin (Vaughan et al., 1997; Jang, 2013). Bs have been reported in three of the four diploid cytotypes (the exception is the most recently evolved cytotype, B7B2, x = 5); Ruiz Rejón et al., 1980; Ebert et al., 1996; Taylor, 1997), and in a range of polyploids (Ebert, 1993; Taylor, 1997). The Bs vary in size and structure between and within cytotypes, and between and within individuals (Ruiz Rejón et al., 1980; Parker et al., 1991; Ebert, 1993; Taylor, 1997).

Recently, molecular tools for analysing the evolution of chromosomes in Prospero have been developed (Jang et al., 2013; Emadzade et al., 2014; Jang & Weiss-Schneeweiss, 2015), which have allowed construction of a phylogenetic framework of the P. autumnale complex. In this study, B-chromosome structure and repeat composition have been analysed in 26 B-carrying plants of diploid and polyploid cytotypes in the complex using 35S and 5S rDNA probes, along with a species-specific and evolutionarily dynamic tandem repeat PaB6 (Emadzade et al., 2014). The degree of amplification of PaB6 and the rDNAs has been compared between the A complement and the accompanying Bs. A recurrent origin of Bs has been established using genomic in situ hybridization (GISH) in B-carrying diploid hybrids. The mode of B meiotic pairing has also been analysed. The results are discussed in the context of de novo origin of Bs in different cytotypes, and in relation to the high amounts of chromosomal restructuring of the regular chromosome sets of P. autumnale.

**Materials and Methods**

**Plant materials**

In total, 26 plants of the *P. autumnale* (L.) Speta complex containing Bs were analysed (Table 1). Fifteen were diploid (three of cytotype AA, eight B7B7, one B6B6, one hybrid AB7, two hybrids B7B2) and 11 were polyploid (three allopolyploids of B6 and B7 origin, and eight autopolyploids of genome B7). For cytological investigations, root meristems were pretreated with a solution of 0.05% colchicine for 4.5 h at room temperature, fixed in ethanol: acetic acid (3:1) for at least 3 h at room temperature and stored at −20°C until use. Young flower buds emerging from the bulb were fixed in ethanol: chloroform: acetic acid (6:3:1) and stored at −20°C.

**Karyotyping and fluorescence in situ hybridization (FISH)**

Chromosome numbers and karyotypes were analysed as described by Jang et al. (2013) using standard Feulgen staining. Chromosomal spreads for FISH were prepared by enzymatic

| Table 1 Plant material of Prospero autumnale complex studied with detailed voucher information |
|---------------------------------------------------------------|
| **Cytotype** | **Locality; collection; accession number** | **2n** |
| **Diploids** | | |
| AA + 1B | Portugal, Peniche; Parker; H549 | 15 |
| AA + 2Bs | Portugal, Peniche; Parker; H560 | 16 |
| AB + 3Bs | Spain, Jaén; Parker; H546 | 17 |
| B7B7 + 1B | Greece, Crete; Speta; H209 | 15 |
| B7B7 + 2Bs | Montenegro; Speta; H415 | 16 |
| B7B7 + 4Bs | Greece, Crete; Speta; H526 | 16 |
| B7B7 + 5Bs | Montenegro; Speta; H412 | 19 |
| B7B7 + 6Bs | Italy, Sicily; Speta; H257 | 20 |
| B7B7 + 1B | Greece, Crete; Speta; H154-1 | 13 |
| B7B7 + 2Bs | Greece, Crete; Weigl; H246 | 15 |
| B7B7 + 8Bs | Greece, Crete; Speta; H525 | 15 |
| **Polyploids** | | |
| B7B7B7B7 + 1B | Greece, Crete; Speta; H213 | 28 |
| B7B7B7B7 + 2Bs | Greece, Crete; Raus; H327 | 30 |
| B7B7B7B7B7 + 1B | Montenegro; Speta; H384 | 29 |
| B7B7B7B7B7 + 3Bs | Spain, Biscay; Parker; H624 | 29 |
| B7B7B7B7B7B7 + 1B | Greece, Crete; Jahn; H339-1 | 36 |
| B7B7B7B7B7B7 + 3Bs | Greece, Karpathos; Raus; H336 | 38 |
| B7B7B7B7B7B7B7 + 4Bs | Greece, Crete; Jahn; H199 | 39 |
| B7B7B7B7B7B7B7B7 + 1B | Greece, Crete; Speta; H536 | 43 |
| B7B7B7B7B7B7B7B7B7 + 3Bs | Tunisia; Speta; H303 | 45 |
| B7B7B7B7B7B7B7B7B7B7 + 4Bs | Tunisia; Speta; H405 | 46 |

1 Material used also for meiotic analyses.
Research 671

Genomic in situ hybridization

Genomic in situ hybridization has been performed in two hybrid individuals, B'B7 (H2A6) and AB7 (H5A6), using parental diploid genomes DNA as probes (Table 1). Total genomic DNA from diploid cytotypes AA, B'B6 and B'B7 was isolated using the CTAB method (Jang et al., 2013) and sheared at 98°C for 5 min. Approx. 1 μg of genomic DNA of each cytotype was labelled using either digoxigenin or biotin nick translation kit (Roche).

Genomic in situ hybridization was carried out following the method described by Jang & Weiss-Schneeweiss (2015) after standard chromosome preparations pretreatment (Jang et al., 2013). The hybridization mix for hybrids containing the B6 genome was modified by addition of unlabelled satellite DNA PaB6 monomers. The satellite DNA PaB6 repeats are present in high copy numbers in cytotype B'B6 and thus, to block these loci and increase GISH performance (noise-to-signal ratio), the excess of unlabelled PaB6 monomers was added to the GISH hybridization mix (Emadzade et al., 2014; Jang & Weiss-Schneeweiss, 2015). Briefly, the hybridization mix included 10% dextran sulphate (Sigma-Aldrich), 0.02 x sodium saline citrate (SSC) buffer, 1% salmon sperm (Sigma-Aldrich), 20 x access of satellite DNA PaB6 (for hybrid B'B6 containing B6 genome) and 3-4 ng μl^-1 of each genomic DNA probe; 10 μl of hybridization mix was applied per slide. After hybridization, slides were washed three times in 2 x SSC at 42°C for 3 min each. Probes were detected as described for FISH. All preparations were analysed with an Axiolmager M2 epifluorescent microscope (Carl Zeiss), and images were captured with a CCD camera and processed using Axiovision v.4.8 (Carl Zeiss) with only those functions that apply to all pixels of the image equally.

Results

The number of Bs varied from one to six per individual, but most frequently a single B chromosome was present. B morphology was variable with acro-, submeta- and metacentrics (Supporting Information Fig. S1). B length also varied, from 1.75 to 4.79 μm (Table 2). B morphology was rather uniform in AA diploids, but varied significantly between plants possessing the B7 genome (Table 2). The most variable Bs were observed in B7 autopolyploids (Table 2).

Tandem repeats in Bs

35S rDNA, 5S rDNA and satellite DNA PaB6 tandem repeats were mapped in mitotic and meiotic chromosomes of the standard complements and in their Bs (Tables 1, 2; Figs 1–3). Hybridization using plastid DNA regions (complete plastid DNA from Vicia faba) produced no discernible signals (Fig. S2).

Satellite DNA PaB6

Satellite DNA PaB6 was found in Bs of diploids and polyploids. Bs in polyploid backgrounds carried higher copy numbers of satDNA and the distribution was more variable (Table 2; Fig. 2). These differences were particularly evident in the B7 genome, in which entire Bs were painted by satellite DNA PaB6 monocots. All five Bs carrying 35S rDNA (in H412) carried Bs with detectable 35S rDNA repeats. All five acrocentric Bs in this plant possessed signals spread over their whole short arms and part of their long arms (Figs 1g, 3 type 3). No secondary constrictions were visible.

5S rDNA, by contrast, was detected in almost all the remaining Bs, with the exception of Bs in two B'B' diploids (Fig. 3). The signals were weak and dot-like in diploids (Figs 1a–j, 3 types 1–7) with the copy number increasing with ploidy level (Figs 1o,p, 3 types 8, 11). Only one B type had a clearly defined and very large 5S rDNA locus. It occupied an extensive pericentric region, which constituted more than half the B (found in allotetraploids and allotetraploids of B6 and B7 genomes; Figs 1p, 3 type 8). 5S rDNA was not detected in the Bs carrying 35S rDNA (Fig. 3 type 3).

In Bs of diploids, copy number and distribution of PaB6 were usually variable. Bs in AA diploids and in the AB7 hybrid were uniform in structure and in tandem repeat distribution. They were enriched in PaB6 (Fig. 3) in subterminal regions of both chromosome arms (Figs 2a–c, 3 type 1a,b) although their amounts differed (Fig. 3). The Bs in the B7 diploid, by contrast, possessed a very low copy number of PaB6 (Figs 2j, 3 type 4), despite the high amount of this repeat in the regular complement (Figs 2j, 3 type 4).

B chromosomes in the B7 diploids were variable (Figs 2d–i, 3 types 2, 3, 5, 7), with the most common type mildly enhanced for PaB6 in the pericentric region but highly enriched.
subterminally in the long arm (types 3, 7). Bs of one B^B^7 plant exhibited very high levels of PaB6 amplification, with nearly the whole B covered (type 5). In plant H209 of B^B^7, a putative, large B showed clear pericentric localization of PaB6 typical of chromosomes of the standard complement, but with a much higher copy number (Figs 2d, 3 type 2); it lacked interstitial and whole B covered (type 5). In plant H209 of B^B^7B^B^7, a putative, B^6B^7 hybrid carried two different B-loci. One B^6B^7 hybrid carried two different B-loci. One B^6B^7 hybrid carried two different B-loci. One B^6B^7 hybrid carried two different B-loci.

### Meiotic behaviour of Bs in diploids

Meiotic behaviour of Bs in two B^7 diploids revealed different patterns of pairing (Fig. 4). In neither did the Bs pair with standard chromosomes. The five Bs in H412, enriched in PaB6 and 35S rDNA, were present as univalents (22 cells; Fig. 4a). By contrast, the two Bs of H415, with PaB6 and 5S rDNA repeats, regularly formed a bivalent (26 cells; Fig. 4b).

### Genomic DNA affinities of Bs

The relationships of Bs to parental genomes were examined using GISH in two different diploid hybrids which were first-generation crosses – B^4B^7 with two Bs (H246; types 5, 6) and

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**Table 2** Basic morphology and length of B chromosomes (Bs) in *Prospero autumnale* complex

| Cytotypes | 2n | 1 | 2 | 3 | 4 | 5 | 6 | Type of Bs | Figures | Accession number |
|-----------|----|---|---|---|---|---|---|------------|--------|-----------------|
| **P. autumnale complex** | | | | | | | | | | |
| Diploids | | | | | | | | | | |
| AA + 1B | 14 + 1B | 2.78^m | 1-1 | 1a, 2a | H549 |
| AA + 1B | 14 + 1B | 2.92^l | 1-1 | – | H623 |
| AA + 2Bs | 14 + 2Bs | 2.05^m | 1.24^m | 1-1 | 1b, 2b | H560 |
| A^B^7 + 3Bs | 14 + 3Bs | 2.49^a | 2.74^a | 2.76^l | 1-2 | 1c, 2c, 4d | H546 |
| B^B^6 + 1B | 12 + 1B | 2.92^l | 4 | 1l, 2j | H174-1 |
| B^B^6 + 2Bs | 13 + 2Bs | 2.55^a | 2.61^a | 5, 6 | 1j, 2k, 4c | H246 |
| B^B^6 + 2Bs | 13 + 2Bs | 3.13^a | 3.33^a | 7 | – | H525 |
| B^B^6 + 1B | 14 + 1B | 3.37^l | 2 | 1d, 2d | H209 |
| B^B^6 + 2Bs | 14 + 2Bs | 2.14^a | 2.20^a | 7 | 4b | H415 |
| B^B^6 + 2Bs | 14 + 2Bs | 2.80^m | 2.93^m | 7 | 1e, 2e | H526 |
| B^B^6 + 4Bs | 14 + 4Bs | 2.50^a | 3.33^a | 3.33^a | 3.33^a | 7 | – | H537 |
| B^B^6 + 4Bs | 14 + 4Bs | 2.71^a | 2.90^a | 2.94^m | 3.20^a | 7 | 1f, 2f | H620 |
| B^B^6 + 5Bs | 14 + 5Bs | 1.81^l | 1.88^a | 1.88^a | 1.92^l | 2.28^l | 3 | 1g, 2g-h, 4a | H412 |
| B^B^6 + 7Bs | 14 + 6Bs | 2.29^m | 2.42^m | 2.42^m | 2.50^m | 2.50^m | 2.71^m | 5 | – | H257 |
| B^B^6 + 7Bs | 14 + 6Bs | 2.07^a | 2.11^a | 2.21^m | 2.22^a | 2.23^m | 2.34^a | 7 | 1h, 2i | H413 |

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1 Chromosome types (indicated in superscript): a, acrocentric; m, metacentric; and s, submetacentric; ^2Bs of these individuals were not measured because it was impossible to identify them among all chromosomes in Feulgen-stained preparations.
AB7 with three Bs (H546; type 1–2). These Bs, therefore, had not undergone meiosis in their current genomic backgrounds. Hybridizations with labelled parental genomic DNAs were carried out.

The Bs in the B6B7 hybrid had a higher affinity for the DNA probe of the B7 than the B6 genome; they also carried PaB6 in subterminal positions on their long arms (Fig. 4c). The Bs of the AB7 hybrid were painted by the A genome alone, although the
Fig. 2 Localization of 5S rDNA (red signals) and satellite DNA PaB6 loci (green signals) in B chromosomes of diploid (a–k) and polyploid (l–u) individuals in the *Prospero autumnale* complex. Metaphase chromosomes were subjected to fluorescence in situ hybridization with 5S rDNA (red in all except for (g) where red depicts 35S rDNA) and 35S rDNA (green). (a) AA + 1B (H549); (b) AA + 2Bs (H560); (c) AA + 3BS (H546); (d) B'B' + 1B (H209); (e) B'B' + 2Bs (H526); (f) B'B' + 4Bs (H620); (g, h) B'B' + 5Bs (H412); (i) B'B' + 6Bs (H413); (j) B'B' + 1B (H154-1); (k) B'B' + 2Bs (H246); (l) B'B'B'B'B' + 1B (H624); (m) B'B'B'B'B'B' + 1B (H384); (n) B'B'B'B'B'B'B'B'B' + 1B (H339-1); (o) B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'B'}
| Type   | DAPI | 5S | 35S | Merged |
|--------|------|----|-----|--------|
| Type 1-1 |      |    |     |        |
| Type 1-2 |      |    |     |        |
| Type 2   |      |    |     |        |
| Type 3   |      |    |     |        |
| Type 4   |      |    |     |        |
| Type 5   |      |    |     |        |
| Type 6   |      |    |     |        |
| Type 7   |      |    |     |        |
| Type 8   |      |    |     |        |
| Type 9   |      |    |     |        |
| Type 10  |      |    |     |        |
| Type 11  |      |    |     |        |

| Cytotype (accession number) |
|-----------------------------|
| AA (H549, H560*, H623)      |
| AB’ (H546)*                 |
| B’B’ (H209)*                |
| B’B’ (H412)*                |
| B’B’ (H154-1)*              |
| B’B’ (H624)                 |
| B’B’ (H246)*                |
| B’B’ (H257)                 |
| B’B’B’B’B’ (H121)          |
| B’B’ (H424)*                |
| B’B’ (H246)*                |
| B’B’ (H413, H415, H526*, H537, H620), B’B’ (H525), B’B’B’B’ (H327), B’B’B’B’ (H159), B’B’B’B’B’ (H303, H405) |
| B’B’B’B’ (H213)*            |
| B’B’ (H384)*                |
| B’B’B’B’ (H339-1)*          |
| B’B’B’B’B’ (H536)*          |

Fig. 3 Repetitive DNA distribution in different types of B chromosomes in the *Prospero autumnale* complex. The individual used as the source of chromosomes depicted in the figure is marked with asterisk. (a) Colocalization of 5S (red) and 35S rDNA (green). (b) Colocalization of 5S rDNA (red) and satellite DNA *PaB6* repeats (green). Whole chromosomes were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; blue). Bars, 1 μm.
intensity of hybridization was slightly lower than that displayed by the standard A chromosomes (Fig. 4d).

Discussion

Structural variation of Bs in the P. autumnale complex

B chromosomes in P. autumnale are, with a single exception, smaller than the smallest standard chromosome (Ainsworth, 1981; Ainsworth et al., 1983; Table 2). Bs, highly variable in structure and heterochromatin composition, have previously been reported in diploids and polyploids of P. autumnale (Ruiz Rejón et al., 1980; Parker et al., 1991; Ebert, 1993; Taylor, 1997) and the Bs in the current study represent a subset of those described. Telocentrics, acrocentrics and metacentrics have previously been documented (Ruiz Rejón et al., 1980; Hong, 1982; Guillén & Ruiz Rejón, 1984; Parker et al., 1991; Ebert et al., 1996), but no telocentrics were included in the current sample. As many as 11 B types were observed based on B morphology but mainly on the distribution of tandem repeats in the Bs (Fig. 3) of the 26 individuals analysed here, representing all available cytotypes. The previous studies of Bs have described numerical and morphological variation using standard Feulgen staining, but heterochromatin content and distribution were established in only two plants (one B6B6, one B7B7; Ebert et al., 1996).

The Bs of the AA cytotype studied here are all rather uniform in structure and nearly identical to the common variant previously reported in AA diploids from Spain and Portugal (Parker et al., 1991). Cytotype B7B7 showed the highest amount of structural B variation, both here and in previous investigations (Ainsworth, 1981; Ebert, 1993; Ebert et al., 1996; Taylor, 1997). While this diversity might simply reflect the higher number of B7B7 plants examined, it may also indicate multiple, independent origins of Bs within the B7 genome lineage, which is widespread across the whole Mediterranean Basin (Vaughan et al., 1997; Jang et al., 2013). Structural polymorphisms of Bs have been reported in many other organisms, both plant and insect (Lopez-Leon et al., 1993; Jones, 1995). In Allium schoenoprasum, for example, 13 different B forms have been described in Welsh populations (Bougourd & Parker, 1979; Holmes & Bougourd, 1989), while in two species of Aster, as many as 29 variants have been identified in studies involving hundreds of populations (reviewed in Jones, 1995).

B chromosomes and repetitive DNA accumulation

Studies of heterochromatin in Bs of two B7B7 plants reported submetacentrics with pericentric dot-like C bands, and acrocentrics with either blocks or dot-like small heterochromatic loci in subterminal, interstitial and/or pericentromeric regions (Ebert, 1993). This corresponds to part of the B variation seen here. Ebert et al. (1996) also reported Bs in B6B6 plants, structurally similar to those found in the current study. These Bs had no C bands, and so agree with observations made here that Bs in the
B6 cytotype have low numbers of tandem repeats of rDNA and PaB6.

Large insertions of plastid and mitochondrial DNA sequences are sometimes detected in plant Bs (Martis et al., 2012; Klemme et al., 2013; Ruban et al., 2014), although none have been found in P. autumnale Bs. Bs frequently share repeat families with the A complement, but also accumulate B-specific families (Langdon et al., 2000; Dhar et al., 2002; Marques et al., 2012; Martis et al., 2012). Bs of P. autumnale share three repeat families with the A complement – 5S and 35S rDNAs and the Prospero-specific satellite DNA PaB6. Although PaB6 is evolutionarily very dynamic and has accompanied diversification of diploid cytotypes and their polyploid derivatives in P. autumnale (Jang, 2013; Emadzade et al., 2014), amplification levels in Bs are not correlated with those in their respective standard complements. No B-specific repeats have yet been found in P. autumnale Bs, although analyses to detect them are under way.

In the phylogenetically well-defined cytotype AA, all Bs have similar repetitive DNA content and distribution, which parallel their structural uniformity. The A genome is geographically restricted to the Iberian Peninsula and may have become isolated from the B7 genome during the most recent glacial era (Parker et al., 1991; Jang et al., 2013). This may have led to the fixation of a single type of B (Parker et al., 1991). By contrast, eight different B-types were found in the widespread B B7 diploid and its derivative polyploids. The five B types found exclusively in B7 autopolyploids might have originated from the most common B type found in diploids, by large-scale amplification of PaB6 and/or 5S rDNA and subsequent divergence.

B chromosomes of polyploids generally had higher PaB6 and 5S rDNA amplification levels than those of diploids, suggesting a positive correlation between these two traits. This may be related to the higher amounts of genome restructuring in polyploids than in diploids within this species complex (Jang, 2013; Weiss-Schneeweiss et al., 2013). No comparable data yet exist for other plant genera.

The lack of meiotic pairing between Bs and standard chromosomes precludes recombination as a direct mechanism mediating spread of PaB6 and rDNA repeats in Bs. Extrachromosomal circular DNAs may facilitate spread and homogenization of tandem repeats (eccDNAs; Cohen et al., 2008; Navratilová et al., 2008), and these should be sought within Prospero. The widespread presence of 5S rDNA repeats in Bs suggests that they originated from standard chromosomes carrying 5S rDNA (chromosome 1 and/or 2; Jang et al., 2013). However, extrachromosomal circular DNAs have again been suggested as a mechanism of spread of this repeat (Cohen et al., 2008, 2010).

On the origin of Bs in P. autumnale

The P. autumnale complex is chromosomally very variable and dynamic so high degrees of variation in structure and repeat composition of Bs are immediately suggestive of their independent and multiple origins. However, structural and repeat content polymorphisms have also been demonstrated in plant groups in which the Bs were shown to be of a single, unique origin (Martis et al., 2012; Marques et al., 2013; Ruban et al., 2014).

The P. autumnale cytotypes are genomically very similar to each other and their repetitive DNA fractions share > 90% similarity (Emadzade et al., 2014; H. Weiss-Schneeweiss et al., unpublished). The standard GISH technique was ineffective in resolving such similar parental genomes, but recently a modified formamide-free GISH technique has allowed clear identification of parental genomes in hybrids (Jang & Weiss-Schneeweiss, 2015). This formamide-free GISH was used in this study to test for the recurrent origin of Bs using two diploid homoploid hybrids – B6B7 (with type 5 Bs) and B7 (with type 1–2 Bs). The results indicated different genomic affinities of these two B types, one similar to the B7 genome (type 5; B6B7 hybrid) and the other to the A genome (type 1–2; AB7 hybrid). It has so far proved impossible to use GISH on Bs in polyploids, because of the high levels of amplification of PaB6 and 5S rDNA. Both repeats produce strong signals which obscure the genomic affinities of Bs even if they are used in high concentrations as unlabelled blocking DNA (Jang & Weiss-Schneeweiss, 2015).

The GISH results give a conservative estimate of two origins of Bs in diploids, one in AA cytotype (type 1) and one in B7 cytotype (type 5); other types could potentially have evolved from type 4 by differential repeat amplification or removal. However, an independent origin is also likely for the B in the B7 (type 4), perhaps indicated by the lack of repeats in this B. This study thus provides the first evidence of recurrent B formation in P. autumnale and investigation will now be extended to the population level.

The genus Prospero has been estimated to originate 6–7 million yr ago (Ma) (Ali et al., 2011), and P. autumnale only c. 1 Ma (K. Emadzade et al., unpublished). Neither Bs nor polyploidy have been found in the two other Prospero species, Prospero obtusifolium and Prospero hanburyi (Jang et al., 2013). Thus, the Bs in Prospero are probably evolutionarily very young. Despite this, the Bs are well established and widespread, and do not pair at meiosis with the regular chromosome complement.

B chromosomes in polyploids of P. autumnale were mainly found in the widespread and common autopolyploids of genome B7, or else in allopolyploids involving B7. Whether these Bs share a common origin or have originated independently and accumulated the same repeats during polyploid genome restructuring will require further study, perhaps using the new formamide-free GISH technique.

Prospero autumnale is a young and chromosomally extremely variable species complex, with diploid cytotypes evolving from an ancestral genome on different evolutionary timescales. It has undergone, and continues to undergo, chromosomal fusions, inversions and translocations accompanied by changes in the repeatome and DNA amounts (Ainsworth et al., 1983; Jang et al., 2013). Bs perhaps represent recent and recurrent by-products of these extensive chromosomal changes, thus following the mode of B-chromosome formation elegantly demonstrated in Secale (Martis et al., 2012) or cichlid fish (Valente et al., 2014). Polyploidy provides an additional level of chromosomal
complexity in *P. autumnale*, which may itself provide a stimulus to genome restructuring and B generation.

**Conclusions**

B chromosomes in the genomically variable species complex *P. autumnale* provide an excellent system in which to address their origin and evolution. The extent of variation of Bs is extraordinarily high and only the minimal level of variation has so far been assessed. The data suggest a recurrent origin of proto-B chromosomes in *P. autumnale*, which are then dynamically evolving. This hypothesis will now be tested on a larger scale, involving population and biogeographical analyses. Analyses of meiotic and postmeiotic behaviour could offer insights into the modes and mechanisms of B transmission.

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**Author contributions**

H.W.-S. and T.-S.J. planned and designed the research. T.-S.J. performed experiments. T.-S.J., J.S.P. and H.W.-S. analysed the data and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Structure of B chromosomes in 24 of 26 analysed individuals of Prospero autumnale.

Fig. S2 Localization of plastid DNA sequences and satellite DNA PaB6 loci in B chromosomes of the Prospero autumnale complex.

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