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DISSERTATION

Chromosomal evolution in *Prospero autumnale* complex

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Tae-Soo Jang

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This thesis is based on following four manuscripts, which are published (one) or prepared as papers for submission in international journals (three).

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T.-S. Jang, K. Emadzade, J. Parker, E.M. Temsch, A.R. Leitch, F. Speta, H. Weiss-Schneeweiss

(**TS-J** carried out the cytogenetic studies, participated in the sequence alignment and drafted the manuscript. KE carried out sequencing of the ITS regions, sequence alignments and analyses, helped to draft the manuscript. JP provided plant material, participated in the design of the study and data interpretation, helped to draft the manuscript. EMT carried out genome size measurements. ARL participated in the design of the study and data interpretation, helped to draft the manuscript. FS provided plant material, helped to draft the manuscript. HW-S designed and coordinated the study, participated in data interpretation, helped to draft the manuscript).

Manuscript 2: Expansion of tandem repeat *PaB6* coincides with chromosomal rearrangements in the chromosomally hyper-variable *Prospero autumnale* complex (Hyacinthaceae)

T.-S. Jang*, K. Emadzade*, J. Macas, P. Novák, A. Kovařík, J. Parker, H. Weiss-Schneeweiss

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Manuscript 3: More than meets the eye: numerical convergence, cycles of hybridization, and contrasting evolutionary trajectories in polyploids of the *Prospero autumnale* complex (Hyacinthaceae)

T.-S. Jang, K. Emadzade, E.M. Temsch, J. Parker, J. Macas, A.R. Leitch, F. Speta, H. Weiss-Schneeweiss

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Manuscript 4: B-chromosomes in *Prospero autumnale* complex (Hyacinthaceae): structural polymorphisms and distinct repeat composition suggest their recurrent origin and ongoing evolution

T.-S. Jang, J. Parker, H. Weiss-Schneeweiss

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ABSTRACT

The circum-Mediterranean *Prospero autumnale* is exceptionally dynamic with respect to karyotype evolution, showing numerical (dysploidy, polyploidy, B-chromosomes) and structural variation. It comprises four evolutionarily distinct diploid cytotypes (AA, B⁷B⁷, B⁶B⁶, and B⁵B⁵), each characterized by a unique combination of basic chromosome number ($x = 5, 6$, and 7), genome size, karyotype structure, and rDNA and satDNA *PaB6* locus distribution. Comparative cytogenetic analyses, interpreted in a phylogenetic context, allowed a model of chromosomal evolution for diploids of *P. autumnale* to be established, with descending dysploidy from $x = 7$ to $x = 6$ and independently from $x = 7$ to $x = 5$ via chromosomal fusions. All cytotypes except B⁵B⁵ participate in polyploidization, without or with hybridization resulting in auto- and allopolyploids. Autopolyploids (only found in genome B⁷) are genomically stable, while allopolyploids are more dynamic. Depending on the divergence of the parental genomes, allopolyploids are either stable with near-complete additivity (polyploids of A and B⁷ origin) or variable both numerically (subgenome compensating aneuploidy, $2n = 25\text{--}28$) and structurally (polyploids of B⁶ and B⁷ origin). In allotetraploids of B⁶ and B⁷ origin, four types of genomically unique and restructured individuals were identified, varying in genome size, rDNA and satellite DNA loci numbers and localization, and copy number of satDNA, in chromosomes of B⁷ origin. These groups represent different cycles of hybridization of the two basic genomes involved: primary allotetraploids (involving B⁶ and B⁷), secondary allotetraploids (primary allotetraploids with B⁷ diploids) and backcrosses of the latter. B-chromosomes and supernumerary segments occur frequently and these are highly variable in structure and repeat composition and are likely to be a by-product of elevated rates of chromosomal rearrangements of the regular chromosome complements. The high incidence of chromosomal changes in all cytotypes of *P. autumnale* strongly contrasts with their morphological near-uniformity, suggesting that chromosomal restructuring is a major mechanism of diversification and eventually speciation in *Prospero*.

Zusammenfassung

Das circum-mediterrane *Prospero autumnale* ist außergewöhnlich variabel im Hinblick auf karyotypische Evolution, die sowohl numerische (Dysploidie, Polyploidie, B-Chromosomen) und strukturelle Variation umfasst. *Prospero autumnale* besteht aus vier distinkten diploiden Zytotypen (AA, B⁷B⁷, B⁶B⁶ und B⁵B⁵), die sich durch spezifische Kombinationen aus Chromosomengrundzahl ($x = 5, 6$ und 7), Genomgröße, Karyotypstruktur und Lokalisierung von rDNA und satDNA *PaB6* auszeichnen. Vergleichende zytogenetische Untersuchungen, interpretiert in einem phylogenetischen Kontext, ermöglichen die Etablierung eines Modelles der chromosomalen Evolution in *P. autumnale*, demzufolge $x = 6$ und $x = 5$ unabhängig voneinander durch Chromosomenfusion aus $x = 7$ entstanden sind (absteigende Dysploidie). Alle Zytotypen außer B⁵B⁵ bilden Polyploide ohne oder mit Hybridisierung (Auto- bzw. Allopolyploide). Während Autopolyploide (nur von B⁷ bekannt) genomisch stabil sind, sind Allopolyploide dynamischer. Abhängig von der Divergenz der parental Genome können Allopolyploide entweder stabil (beinahe völlige Additivität: Polyploide von A und B⁷) oder numerisch oder strukturell variabel sein (Polyploide von B⁶ und B⁷; Kompensierende Aneuploidie auf Subgenomebene, $2n = 25-28$). In Allotetraploiden aus B⁶ und B⁷ wurden vier Typen identifiziert, die sich hinsichtlich Genomgröße, Lokalisierung und Anzahl von rDNA und satDNA Loci sowie der Kopienanzahl von satDNA in den B⁷-Chromosomen unterscheiden. Diese Gruppen sind das Ergebnis mehrerer Hybridisierungszyklen der beiden beteiligten Genome: primäre Allotetraploide (B⁶ mit B⁷), sekundäre Allotetraploide (primäre Allotetraploide mit B⁷) und Rückkreuzungen. B-Chromosomen und überzählige chromosomale Segmente, die häufig und in großer Vielfalt (bezüglich Struktur und Komposition repetitiver Elemente) auftreten, sind wahrscheinlich das Nebenprodukt erhöhter Raten chromosomaler Umstrukturierung des regulären Chromosomensatzes. Das häufige Auftreten chromosomaler Änderungen in allen Zytotypen von *P. autumnale* steht in starkem Widerspruch zur geringen morphologischen Variabilität und deutet darauf hin, dass chromosomale Evolution den Hauptmechanismus der Diversifikation und Speziation in *Prospero* darstellt.

General Introduction

Genome evolution in plants

Eukaryotic chromosomes are the organized linear structures that carry the majority of the genetic material of the organism and are located within nuclei of all eukaryotic cells. They differ in size, shape and composition of their DNA and proteins (Schubert, 2007). All of these features can be affected during evolution and partake in chromosomal diversification, accompanying taxa divergence and eventually speciation (Stebbins, 1971; Rieseberg, 2001; Levin, 2002; Schubert, 2007; Leitch and Leitch, 2008). Chromosomal rearrangements involving inversions, translocations, duplications, deletions, fusions and fissions result in changes in chromosome number (dysploidy and polyploidy) and chromosome structure (Schubert, 2007; Lysák and Schubert, 2013). Genome size changes and more subtle changes in sequence composition of the repetitive fraction of the genome, most commonly involving expansions or reductions of repetitive DNA sequence amounts, constitute additional sources of variation occurring during the evolutionary history of taxa (Weiss-Schneeweiss and Schneeweiss, 2013).

Among all genomic changes, polyploidy, or whole-genome duplication (WGD), is one of the most prominent features accompanying evolution and diversification of new taxa in higher plants (Stebbins, 1971; Soltis et al., 2009; Weiss-Schneeweiss et al., 2013). Two types of polyploids are commonly recognized: autopolyploids originating via multiplication of chromosome sets (genomes) from within a single species and allopolyploids produced via hybridization of related genomes or species followed or accompanied by multiplication of parental chromosome sets. Both arise as a result of failure of cell division either during mitosis or meiosis (Stebbins, 1971; Otto and Whitton, 2000; Ramsey and Schemske, 2002).

Polyploidy has occurred frequently in angiosperm history at different evolutionary scales, with virtually all angiosperms being of ancient paleopolyploid origin (Soltis et al., 2009; Husband et al., 2013). Polyploidy has been estimated to have been involved in 15% of speciation events of angiosperms, and up to 23% in monocots (Wood et al., 2009). Historically, autopolyploidy has been considered as less frequent and less important than allopolyploidy (Stebbins, 1971). Recently studies have, however, clearly shown that natural autopolyploids are common in many plant groups (Ramsey and Schemske, 1998, 2002; Soltis et al., 2007; Parisod et al., 2010; Husband et al., 2013; Krejčíková et al., 2013) and might confer evolutionary advantages (Soltis et al., 2007).

The evolution of repetitive DNA in plant genomes

Higher plant genomes are densely populated by various types of repetitive DNAs that are present in hundreds or thousands of copies (Heslop-Harrison and Schwarzhacher, 2011). Recent methodological advances have greatly advanced our understanding of genome composition and evolution, allowing broader inferences to be made of evolutionary trends within angiosperms. Detailed physical chromosomal maps, which enable evolutionary patterns and processes to be determined, can be constructed using fluorescence *in situ* hybridization (FISH) with single copy and repetitive DNAs. Repetitive DNAs encompass tandem repeats, both coding (rDNAs) and non-coding (species- or genus-specific satellite DNAs), and dispersed repeats with mobile genetic elements. All of these DNA types are used for detailed chromosome and genome mapping (Schwarzhacher and Heslop-Harrison, 2000; Kulikova et al., 2001; Cuadrado and Jouve, 2002; Mishima et al., 2002; Pedrosa et al., 2002; Lysák et al., 2006; Kotseruba et al., 2010; Weiss-Schneeweiss and Schneeweiss, 2013). Patterns of gross chromosomal evolution have been well established in several economically important plant genera, including *Nicotiana* (Lim et al., 2000, 2007; Clarkson et al., 2005) and *Beta* (Schmidt and Heslop-Harrison, 1996), as well as in model organisms and their wild relatives (e.g., Brassicaceae, Lysák et al., 2006; Mandáková et al., 2012). Comparative evolutionary cytogenetics of wild plant groups has so far been much less explored (but see *Hepatica*, Weiss-Schneeweiss et al., 2007; *Anemone*, Mlinarec et al., 2012; *Melampodium*, Weiss-Schneeweiss et al., 2012). Chromosomal evolution has also been studied in polyploids often indicating higher dynamic of genome evolution in polyploids in comparison to their diploid progenitors (*Nicotiana*, Clarkson et al., 2005; *Tragopogon*, Chester et al., 2012).

5S and 35S rDNAs

Tandemly repeated genes encoding 35S (18S-5.8S-25S) and 5S rRNAs have been particularly useful for inferring relationships between closely related wild species for which very scarce or no genomic data were available (Lim et al., 2000; Weiss-Schneeweiss et al., 2008; Kotseruba et al., 2010; Mlinarec et al., 2012; Weiss-Schneeweiss et al., 2012). The 35S rDNA unit, coding for 18S, 5.8S, and 25S rRNAs, also includes external transcribed spacer (ETS), two internal transcribed spacers (ITS1 and 2) and the non-transcribed intergenic spacer (IGS). Numerous 35S rDNA repeats are tandemly arranged in one or more loci in the genome known as nucleolar-organizer regions (NORs). The 5S rDNA repeat encompasses c. 121 bp long genic region and variable non-transcribed spacer (NTS). The tandem arrays of those repeats mostly map independently of 35S rDNA loci in plant chromosomes (Małuszyńska et al., 1998; but see also Garcia et al., 2010; Garcia and Kovařík, 2013). Copy

numbers of 5S rDNA in the genome are usually lower than those of 35S rDNA (Lapitan et al., 1991; Sastri et al., 1992). Since the coding regions of these two markers are highly conserved across large evolutionary timescales, even between eukaryotes and prokaryotes (Heslop-Harrison, 2000; Weiss-Schneeweiss et al., 2008), their locations can easily be detected and provide useful landmarks for chromosome identification. Additionally, the nucleotide sequences of 5S rDNA NTS and both ITS regions of 35S rDNA are often used for inferring phylogenetic relationships of analyzed taxa. The combination of such genetic and cytogenetic approaches allows direct interpretation of rDNA loci number and distribution in a phylogenetic context (Weiss-Schneeweiss et al., 2007, 2008, 2012; Mahelka et al., 2013).

Tandemly repeated non-coding satellite DNA sequences

The repetitive non-coding genome fraction consists of many families of dispersed repetitive elements (mostly retroelements and DNA transposons) and tandemly repeated DNA sequences (telomeres, various satellite DNAs). These elements can range in size from 2 bp to more than 10 kbs (Heslop-Harrison, 2000; Macas et al., 2007; Weiss-Schneeweiss and Schneeweiss, 2013). Tandemly repeated satellite DNAs are almost universally present in eukaryotic genomes and are very dynamic (Ugarković and Plohl, 2002). Changes in copy number and chromosomal location, as well as sequence divergence over evolutionary time, facilitates comparative evolutionary analyses of closely related plant genomes (Lim et al., 2000; Cuadrado and Jouve, 2002; Navrátilová et al., 2003; Pires et al., 2004; Rosato et al., 2012). The genomic localization and diversification of tandem repeats has been shown to be very dynamic in most groups of related taxa (Cuadrado and Jouve, 2002; Menzel et al., 2008; Koukalova et al., 2010; Rosato et al., 2012). Polyploids, particularly allopolyploids, often exhibit elevated rates of repeat evolution in comparison to their diploid progenitors, experiencing loss, amplification or formation of novel repeats (Renny-Byfield et al., 2011). These processes are often directional and influence one of the parental genomes more than the other (Buggs et al., 2012).

Supernumerary genetic material: B-chromosomes and chromosomal segments

Supernumerary genetic materials are dispensable components of the genome, most frequently present as B-chromosomes but also found as supernumerary chromosomal segments. B-chromosomes have been reported in about 8% of monocots and 3% of eudicots in angiosperms, in numerous species of animals, and in fungi (Jones, 1995; Camacho et al., 2000; Levin et al., 2005; Houben et al., 2013). The frequency of supernumerary segments is unclear, but they occur in animals and plants (Ruiz Rejón et al., 1980; Wilby and Parker, 1988; Parker et al., 1991; Camacho et al., 2000). B-chromosomes may contain specific

satellite DNAs, ribosomal DNA sequences, microsatellite DNA, and transposable elements (Małuszyńska and Schweizer, 1989; Donald et al., 1995; Camacho, 2005; Peng and Cheng, 2011).

The origin and evolution of B-chromosomes in plants has recently been subject to investigations using molecular technologies (Camacho et al., 2000; Dhar et al., 2002; Hasterok et al., 2002; Peng and Cheng, 2011; Banaei-Moghaddam et al., 2012; Martis et al., 2012). B-chromosomes were inferred to originate from standard chromosomes following multiple chromosomal rearrangements (segmental or whole-genome duplications, unequal translocations, and insertions), during species differentiation and evolution, often associated with chromosome number reduction (Jones et al., 2008; Martis et al., 2012). Once established, B-chromosomes evolve by accumulation of additional standard A-chromosome-derived sequences, including various types of repeats, and organellar genomes, as well as via formation and amplification of B-chromosome specific repeats (Martis et al., 2012; Banaei-Moghaddam et al., 2012).

The study system

The genus *Prospero* presents a particularly suitable system for analyzing the role of chromosomal change in plant diversification and speciation. Up to 14 species have been proposed in the genus in various taxonomic treatments (see Appendix 1; Battaglia, 1964; Hong, 1982; Ainsworth et al., 1983; Parker et al., 1991; Ebert et al., 1996; Vaughan et al., 1993, 1997; Speta, 2000; Brullo et al., 2009; Hamouche et al., 2010), but only two species and a species-complex are now commonly recognized as comprising the genus. Both *P. obtusifolium* (Poir.) Speta ($x = 4$) and *P. hanburyi* (Baker) Speta ($x = 7$) are morphologically identifiable and chromosomally stable diploids. By contrast, the species-complex referred to as *P. autumnale* (L.) Speta is chromosomally very dynamic but shows little morphological diversity (Ebert et al., 1996; Vaughan et al., 1997; Jang et al., 2011).

The species complex of *P. autumnale* exhibits exceptionally high levels of chromosomal variation, with three basic chromosome numbers ($x = 5, 6$, and 7) and many ploidy levels ($2x$ to about $20x$) of both auto- and allopolyploid type (Ainsworth et al., 1983; Ebert et al., 1996; Vaughan et al., 1997). Four diploid cytotypes (AA , B^7B^7 , B^6B^6 , and B^5B^5), with different basic chromosome numbers and distinctive karyotypes, have been described in the complex. The B^7B^7 cytotype ($2n = 14$) is most widespread and circum-Mediterranean, the AA cytotype ($2n = 14$) occurs in countries bordering the Atlantic Ocean in Iberia and North Africa and has a karyotype structure very similar to B^7B^7 , but with a genome size nearly 70% larger (Ainsworth et al., 1983; Vaughan et al., 1997). It has been proposed that two cytotypes with

reduced chromosome numbers originated via descending dysploidy from $x = 7$ by two successive fusion events. Fusion of chromosomes 6 and 7 resulting in a large submetacentric fusion chromosome gave rise to cytotype B^6B^6 ($2n = 12$) endemic to Crete, while a further fusion involving chromosomes 1 and 3 from within B^6B^6 cytotype was proposed for the origin of the B^5B^5 cytotype ($2n = 10$) endemic to Libya (Taylor, 1997; Vaughan et al., 1997). A myriad of autopolyploids (encountered only in genome B^7) on various ploidy levels ($4x$ and $6x$ prevailing) and allopolyploids of two genomic combinations: (i) A and B^7 ($4x$, $6x$) and (ii) B^6 and B^7 (again mostly with tetraploids and hexaploids) occur frequently as natural populations, along with occasional odd-numbered polyploid individuals, such as $3x$ ($B^6B^6B^7$ and $B^6B^7B^7$) and $5x$ ($B^7B^7B^7B^7B^7$ and $B^6B^6B^7B^7B^7$) (Ainsworth et al., 1983; Parker et al., 1991; Vaughan et al., 1997; Jang et al., 2011).

Molecular phylogenetic relationships within the family Hyacinthaceae have been inferred from plastid DNA sequence data (Pfosser and Speta, 1999; Pfosser et al., 2003; Ali et al., 2012). These studies, however, have included only one or two accessions of *Prospero* without the essential cytological information. The age of the divergence one species of the genus - *P. obtusifolium* - has been inferred as 6.43 my (Ali et al., 2012).

Aims of the study

A thorough investigation of the cytological features of the chromosomally remarkably variable species complex of *P. autumnale* employing repetitive DNA fractions, and the interpretation of their changes in a sound phylogenetic context, has allowed us to address significant evolutionary questions concerning the origins, mechanisms, directions and frequencies of chromosomal changes. The role of chromosome change in race formation and taxa differentiation can thus be firmly established. Phylogenetic, comparative cytogenetic and molecular analyses using three types of repeats - 5S, 35S rDNA and a genus-specific satellite DNA - have been employed in an attempt to understand the detailed genome evolution of *Prospero* itself, and the *P. autumnale* complex in particular.

Inference of the evolutionary significance of chromosomal changes for diploid cytotype differentiation focuses on: (1) directionality of and mechanisms involved in, dysploid base chromosome numbers change at the diploid level interpreted in a firm phylogenetic context, and (2) the dynamics of genome size and chromosome structure changes through the identification of genome components involved in these changes.

The occurrence of polyploidy has been well documented in the *P. autumnale* complex, but assessments of evolutionary dynamics have not been made. Thus, this thesis addresses

questions about genomic consequences and the evolutionary potential of polyploidy in *P. autumnale*: (1) the dynamics of evolutionary changes of auto- and allopolyploid genomes of different origins and the factors influencing those; (2) the directionality of genome evolution dependent on parental origin.

The high frequency of structurally variable B-chromosomes documented in the genus raises questions about their composition, the patterns and dynamics of their evolution, and their origin: (1) the extent and patterns of variation of B-chromosomes indicated by their DNA composition in different diploid and polyploid cytotypes; (2) the potential multiple origins of B-chromosome systems and their establishment in early stages of evolution; (3) the distinctive evolution of B-chromosomes in genomes at different ploidy levels.

The multi-levelled chromosomal variation documented here in the genus *Prospero* including dysploidy, polyploidy, genome size and chromosomal structure changes as well as the presence of accessory genetic material raises questions about the significance of all these changes for taxa divergence and eventually speciation. Thus, the overall chromosomal variation interpreted in a phylogenetic context has been compared to gross morphological differentiation, to infer whether chromosomal change might be assigned a direct and perhaps leading role in the evolution of the genus.

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RESEARCH ARTICLE

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Chromosomal diversification and karyotype evolution of diploids in the cytologically diverse genus *Prospero* (Hyacinthaceae)

Tae-Soo Jang¹, Khater Emadzade¹, John Parker², Eva M Temsch¹, Andrew R Leitch³, Franz Speta⁴ and Hanna Weiss-Schneeweiss^{1*}

Abstract

Background: *Prospero* (Hyacinthaceae) provides a unique system to assess the impact of genome rearrangements on plant diversification and evolution. The genus exhibits remarkable chromosomal variation but very little morphological differentiation. Basic numbers of $x = 4, 5, 6$ and 7 , extensive polyploidy, and numerous polymorphic chromosome variants were described, but only three species are commonly recognized: *P. obtusifolium*, *P. hanburyi*, and *P. autumnale* s.l., the latter comprising four diploid cytotypes. The relationship between evolutionary patterns and chromosomal variation in diploids, the basic modules of the extensive cytological diversity, is presented.

Results: Evolutionary inferences were derived from fluorescence *in situ* hybridization (FISH) with 5S and 35S rDNA, genome size estimations, and phylogenetic analyses of internal transcribed spacer (ITS) of 35S rDNA of 49 diploids in the three species and all cytotypes of *P. autumnale* s.l. All species and cytotypes possess a single 35S rDNA locus, interstitial except in *P. hanburyi* where it is sub-terminal, and one or two 5S rDNA loci (occasionally a third in *P. obtusifolium*) at fixed locations. The localization of the two rDNA types is unique for each species and cytotype. Phylogenetic data in the *P. autumnale* complex enable tracing of the evolution of rDNA loci, genome size, and direction of chromosomal fusions: mixed descending dysploidy of $x = 7$ to $x = 6$ and independently to $x = 5$, rather than successive descending dysploidy, is proposed.

Conclusions: All diploid cytotypes are recovered as well-defined evolutionary lineages. The cytogenetic and phylogenetic approaches have provided excellent phylogenetic markers to infer the direction of chromosomal change in *Prospero*. Evolution in *Prospero*, especially in the *P. autumnale* complex, has been driven by differentiation of an ancestral karyotype largely unaccompanied by morphological change. These new results provide a framework for detailed analyses of various types of chromosomal rearrangements and karyotypic variation in polyploids.

Keywords: Chromosomal evolution, FISH, Genome size, Hyacinthaceae, ITS, Phylogeny, *Prospero*, rDNA

Background

Chromosomal change plays an important role in plant evolution, diversification, and speciation [1,2]. When carried out against a phylogenetic background [1,3-5] comparative analyses of karyotypes allow inferences regarding evolutionary history.

Detailed physical chromosomal maps, which enable evolutionary patterns and processes to be determined,

can be constructed using FISH (fluorescence *in situ* hybridization) from both single copy and repetitive DNAs, such as rDNA, species- or genus-specific repetitive DNAs, individual chromosome DNAs [1,6-10]. Patterns of chromosomal evolution using FISH have been established in several economically important plant genera (e.g., *Nicotiana* [3,11], *Beta* [12]) as well as in model organisms and their wild relatives (e.g., Brassicaceae [1,13]). Comparative evolutionary cytogenetics of wild plant groups, however, has been much less explored (e.g., *Hepatica* [14], *Anemone* [15], *Melampodium* [16]).

* Correspondence: hanna.schneeweiss@univie.ac.at

¹Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030, Vienna, Austria

Full list of author information is available at the end of the article

The markers of choice for cytogenetic evolutionary studies include tandemly repeated genes encoding 5S and 35S rRNA within the nucleus. The 35S rDNA loci (18S–5.8S–25S rDNA) are located in the nucleolar-organizer regions (NORs), while tandem arrays of 5S rDNA map independently of them (but see [17]). Copy numbers of 5S rDNA are usually lower than 35S rDNA [18,19]. Since the coding regions of these two markers are conserved across large evolutionary units [4,20] their localization provides useful landmarks for chromosome identification [20–22]. Partial DNA sequences of these two rDNA types (e.g., ITS of 35S rDNA or NTS of 5S rDNA) are also commonly used for inferring phylogenies [16,23]. This allows the interpretation of cytological information in a strict phylogenetic context, giving detailed insights into the patterns of evolution of genomes.

A particularly suitable system for analyzing the role of chromosomal change in plant diversification and speciation is provided by the genus *Prospero* Salisb. (Hyacinthaceae). This genus is distributed around the whole Mediterranean basin, north to Britain and Russia (Figure 1). Across this area *Prospero* exhibits exceptionally high levels of chromosomal variation, with basic chromosome numbers of $x = 4, 5, 6$, and 7 , alongside levels of ploidy up to about 20-fold [24–26]. Three species are commonly recognized in the genus: *P. obtusifolium* (Poir.) Speta ($x = 4$) and *P. hanburyi* (Baker) Speta ($x = 7$), both chromosomally stable, and a dynamic species complex referred to as *P. autumnale* (L.) Speta. Within *P. autumnale*, up to 15 smaller, local, segregates have been described [27–33], but these are only subtly differentiated morphologically (quantitative differences

and distinct chromosome numbers/ploidy levels, [32]). Thus, in this paper, we recognize only the three species as comprising *Prospero* for the clarity of the data interpretation. The relationship of genomic, chromosomal, and phylogenetic analyses to species delimitation and their correlation with distinct morphological characters will only emerge from broader evolutionary studies of the genus.

P. obtusifolium ($x = 4$) and *P. hanburyi* ($x = 7$) are morphologically distinct entities found within the range of the *P. autumnale* complex, the former two being geographically restricted to the western Mediterranean and to the Levant respectively. They are known only as diploids. By contrast, the *P. autumnale* complex exhibits a spectacular array of genomic and chromosomal variation, unparalleled in any other flowering plant, with multiple basic chromosome numbers, a huge range of levels and complexity of polyploidy, and a spectacular array of chromosomal polymorphisms (including supernumerary segments, B-chromosomes, and inversions). Four distinct diploid cytotypes with basic chromosome numbers of $x = 5, x = 6$, and two with $x = 7$, have so far been described [24].

The two $x = 7$ diploid cytotypes are referred to as AA and B⁷B⁷, with AA found only in countries bordering the Atlantic Ocean in Iberia and North Africa and B⁷B⁷ occupying the countries around the Mediterranean Basin and on its islands; they overlap only in Spain [25]. The karyotype morphologies of AA and B⁷B⁷ are very similar, but differ significantly in chromosome size and DNA amount, and, more trivially, in the location of the single NOR within chromosome 3 [25,26,34]. Cytotype B⁷B⁷

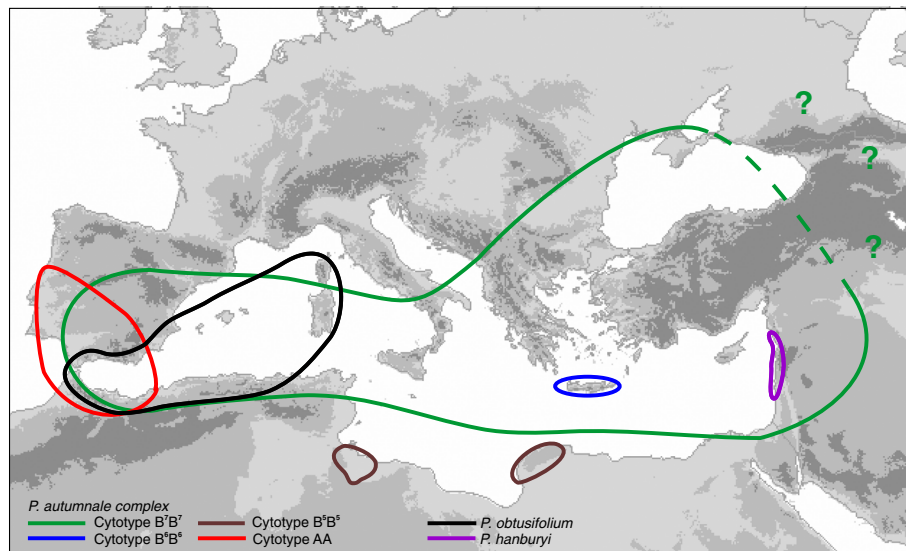


Figure 1 Map of distribution of diploid species and cytotypes of the genus *Prospero*. Dashed line in the eastern range of distribution of cytotype B⁷B⁷ and question marks indicate incomplete information on the distribution of this cytotype.

has been hypothesized to be most similar to the ancestral karyotype of the complex [26].

Diploid plants based on $x=6$ (cytotype B^6B^6) are endemic to Crete. The B^6B^6 karyotype carries a large submetacentric chromosome referred to as $F^1(6-7)$ [F = fusion/fission; numbers in parentheses indicate chromosomes proposed to be involved in fusion/fission], while the remaining chromosomes correspond closely in morphology and homoeology to chromosomes 1–5 of the B^7/A genomes. A diploid of constitution B^5B^5 is endemic to Libya [26] and carries two fission/fusion chromosomes, assigned to as $F^2(6-7)$ and $F^3(1-3)$, with respect to the karyotype of A/B^7 genome [24,26].

Despite the enormous chromosomal and DNA amount variation within the *P. autumnale* complex, there is no large-scale accompanying morphological differentiation. The mechanisms involved in chromosome change and its directionality, might therefore allow us to infer evolutionary patterns within the genus. Within *P. autumnale*, we have previously [24,35,36] attempted to establish phylogeny from chromosome numbers and karyotype structure supplemented by analyses of meiotic configurations in diploid hybrids. Two sequential chromosomal fusions were proposed for the reduction of the chromosome number from $x=7$ (AA , B^7B^7) to $x=6$ (B^6B^6) and $x=5$ (B^5B^5) [26]. In addition to this descending dysploid shift, genome size also varies, with a major discontinuity between genomes B^7 , B^6 and B^5 and the large genome A [26,37]. No evolutionary directionality has been ascribed to this change.

Phylogenetic relationships within the family Hyacinthaceae have been inferred from plastid DNA sequence analyses [38–40]. These studies, however, included only one or two accessions of *Prospero* (of unknown ploidy levels), so no assessment of phylogenetic relationships within the genus could be made. This present study provides the first comprehensive analysis of phylogenetic relationships among all the diploids identified in the genus *Prospero*, based on karyotype and genome size changes, analyzed against a rigorous DNA phylogeny, allowing previous hypotheses concerning karyotypic evolution to be tested. This study provides also a framework for studying evolutionary patterns in polyploid genomes of *Prospero*.

The aims of this study are to: (1) establish numbers and locations of 5S and 35S rDNA loci in all diploid species and cytotypes of *Prospero*; (2) analyze the evolution of rDNA loci and genome size in a phylogenetic context; (3) test previous hypotheses concerning the evolution of basic chromosome number in the *P. autumnale* complex; and (4) propose a new model for chromosomal rearrangements within the genus and to evaluate their role in the diversification of taxa.

Results

Chromosome numbers and karyotype structure in the genus *Prospero*

Chromosome counts confirmed all chromosome numbers reported earlier for diploids in the genus *Prospero*: $2n=8$, 10, 12, and 14 (Table 1, Figure 2).

Prospero obtusifolium

All six plants of *P. obtusifolium* were diploid with $2n=2x=8$ (Table 1, Figure 2). The karyotype consisted of three pairs of submetacentric and one pair of subtelocentric chromosomes (Figure 2) with Haploid Karyotype Length (HKL) of $29.01 \pm 0.77 \mu\text{m}$ (Table 2). A single nucleolar-organizing region (NOR) was localized within the pericentric region of the short arm of chromosome 3 (Figure 2). The 1C DNA amount of *P. obtusifolium* was $4.94 \pm 0.039 \text{ pg}$ (Table 2).

Prospero hanburyi

The three plants of *P. hanburyi* were diploid with $2n=2x=14$ (Table 1, Figure 2), comprising four pairs of near-metacentric and three pairs of submetacentric chromosomes (Figure 2). The HKL was $44.90 \pm 4.04 \mu\text{m}$ (Table 2). A single NOR was localized subterminally on the short arm of chromosome 2 (Figure 2). This contrasted to the interstitial localization of NORs in all other *Prospero* taxa and cytotypes. The 1C content of *P. hanburyi* was $6.81 \pm 0.017 \text{ pg}$ (Table 2). The karyotypes of these two species showed little structural similarity to the diploid karyotypes within the *P. autumnale* complex (Figure 2).

The *Prospero autumnale* complex

The four diploid cytotypes (AA , B^5B^5 , B^6B^6 , and B^7B^7) of the *P. autumnale* complex differed not only in basic chromosome number, but also in karyotype structure due to fusion/fission and genome size (Table 1, Figure 2).

Cytotype AA ($2n=2x=14$)

In all six individuals the karyotype consisted of five sub-metacentrics (chromosomes 1–3 and 5–6), one subtelocentric (chromosome 4), and one near-metacentric (chromosome 7; Figure 2). The HKL was $48.35 \pm 7.15 \mu\text{m}$ (Table 2) with a 1C DNA content of $7.85 \pm 0.045 \text{ pg}$ (Table 2). A single NOR was adjacent to the centromere in the long arm of chromosome 3 (Figure 2).

Cytotype B^7B^7 ($2n=2x=14$)

The karyotype in seventeen individuals consisted of five sub-metacentrics (chromosomes 1–3 and 5–6), one subtelocentric (chromosome 4), and one near-metacentric (chromosome 7), each identifiable by size and morphology (Table 1 and Figure 2).

Table 1 Plant material studied with localities, chromosome numbers, and GenBank accession numbers of ITS DNA sequences

Cytotype	Voucher information (accession number)	2n	ITS GenBank accession number
Outgroups			
<i>Dipcadi</i> sp.	cult. HBV (H336)	-	KC899267
<i>Othocallis siberica</i> (Haw.) Speta	cult. HBV (H2159)	12	KC899268
Genus <i>Prospero</i> Salisb.			
<i>P. obtusifolium</i> (Poir.) Speta	Spain, Parker s.n., cult. HBV (H540)	8	KC899275
	Morocco, Parker 15500-1, cult. HBV (H547)	8	KC899273
	Spain, Parker DL20, cult. HBV (H556)	8	KC899276
	Spain, Parker DL8, cult. HBV (H559)	8	KC899272
	Morocco, Parker 15607, cult. HBV (H563)	8	KC899277
	Morocco, Parker 15607, cult. HBV (H564)	8	KC899274
<i>P. hanburyi</i> (Baker) Speta	Turkey, Findikpinar A, Leep s.n., cult. HBV (H115)	14	KC899269
	Turkey, Narlikuyu, Silifke, 475/01, cult. HBV (H231)	14	KC899270
	Turkey, Findikpinar, L75/T25, cult. HBV (H397)	14	KC899271
<i>P. autumnale</i> (L.) Speta s.l.			
AA	Spain, Huelva, Parker s.n., cult. HBV (H541)	14	KC899278
	Spain, Badajoz, Parker CV3, cult. HBV (H543)	14	KC899279
	Spain, Huelva, Parker s.n., cult. HBV (H548)	14	KC899280
	Portugal, Peniche, Parker s.n., cult. HBV (H550)	14	KC899281
	Portugal, Peniche, Parker s.n., cult. HBV (H551)	14	KC899283
	Spain, Huelva, Parker s.n., cult. HBV (H557)	14	KC899282
B ⁷ B ⁷	Greece, Crete, Speta KR245, cult. HBV (H47)	14 ¹	KC899309
	Greece, Peloponnisos, Speta 81, cult. HBV (H74)	14 ¹	KC899308
	Greece, Rhodos, Faliraki, Speta 52800, cult. HBV (H137)	14 ²	KC899296
	Höner, s.n., cult. HBV (H228)	14 ²	KC899295
	Cyprus, Speta 53872, cult. HBV (H239)	14 ¹	KC899297
	Greece, Samos, Tod 52684, cult. HBV (H241)	14 ¹	KC899310
	Montenegro, Speta s.n., cult. HBV (H422)	14 ²	KC899302
	Montenegro, Speta s.n., cult. HBV (H424)	14 ²	KC899305
	Italy, Sicily, Speta 51990, cult. HBV (H428)	14 ¹	KC899298
	Greece, Crete, Speta KR 15, cult. HBV (H440)	14 ¹	KC899306
	Speta 52746, cult. HBV (H447)	14 ¹	KC899299
	Greece, Kalamitsi, Speta 52690, cult. HBV (H450)	14 ¹	KC899311
	Greece, Crete, Speta s.n., cult. HBV (H460)	14 ¹	KC899307
	Greece, Naxos, Speta 3, cult. HBV (H575)	14 ¹	KC899300
	Serbia, Siget-Baun, Rat s.n., cult. HBV (H576)	14 ²	KC899303
	Ukraine, Nikita, Roman RK4-1, cult. HBV (H591)	14 ²	KC899304
	Israel, Nene Han, Parker, s.n., cult. HBV (H612)	14 ¹	KC899301
B ⁶ B ⁶	Greece, Crete, Speta KR20, cult. HBV (H158)	12	KC899289
	Greece, Crete, Speta CR95-99, cult. HBV (H166)	12	KC899284
	Greece, Crete, Speta 95-99, cult. HBV (H170)	12	KC899285
	Greece, Crete, Speta KR20, cult. HBV (H195)	12	KC899290
	Greece, Crete, Jahn 854, cult. HBV (H197)	12 ⁴	KC899286

Table 1 Plant material studied with localities, chromosome numbers, and GenBank accession numbers of ITS DNA sequences (Continued)

B ⁵ B ⁵	Greece, Crete, Speta 52635, cult. HBV (H274)	12	KC899291
	Greece, Crete, N.B. 6890, cult. HBV (H340)	12 ³	KC899287
	Greece, Crete, Jahn 353, cult. HBV (H408)	12	KC899288
	Greece, Crete, Jahn & Böhling 9131Z, cult. HBV (H427)	12	KC899293
	Greece, Crete, Speta CR95-99, cult. HBV (H468)	12	KC899292
	Greece, Crete, Speta 52613, cult. HBV (H520)	12	KC899294
	Libya, Mt. Tobi, Parker s.n., cult. HBV (H566)	10	KC899313
	Libya, Mt. Tobi, Parker To-2, cult. HBV (H581)	10	KC899314
	Libya, Mt. Tobi, Parker To-28, cult. HBV (H582)	10	KC899316
	Libya, Mt. Tobi, Parker s.n., cult. HBV (H631)	10	KC899317
	Libya, Mt. Tobi, Parker s.n., cult. HBV (H637)	10	KC899312
	Libya, Nagasa, Parker s.n., cult. HBV (H640)	10	KC899315

¹One locus of 5S rDNA.

²Duplication of 5S rDNA locus in chromosome 1.

³Translocation of NOR of one on the homologous chromosomes 3 to chromosome F¹(6–7).

⁴Translocation of both NORs to chromosome F¹(6–7).

Plant material is in cultivation in Botanical Garden of the University of Vienna (HBV). Each individual in cultivation has a unique ID (in brackets, e.g., H336).

The karyotypes of A and B⁷ genomes were extremely similar in morphology and the numbers indicated homoeologies. HKL and 1C DNA contents have been established in selected individuals, which differed in their 5S rDNA locus number (for details see below). The HKLs were $28.70 \pm 1.74 \mu\text{m}$ and $33.76 \pm 1.45 \mu\text{m}$ while genome sizes were $4.45 \pm 0.023 \text{ pg}$ and $4.23 \pm 0.048 \text{ pg}$ respectively (Table 2). A single NOR was adjacent to the centromere in the long arm of chromosome 3 (Figure 2). Cytotype B⁷B⁷ is the most widespread in *P. autumnale*.

Cytotype B⁶B⁶ (2n = 2x = 12)

In all eleven bulbs, the karyotype consisted of four sub-metacentrics (chromosomes 1–3 and 5), one sub-telocentric (chromosome 4), and one large sub-metacentric presumptive fusion chromosome classified as chromosome number F¹(6–7). Chromosome numbering again reflects homoeology to B⁷ and A genomes (Figures 2 and 3, Additional file 1: Figure S1).

In nine individuals, both NORs were located in the long arm of the chromosome homoeologous to chromosome 3, although in a more median position (Figure 2). The other two individuals (from different populations) have apparently undergone translocation of one or both NOR regions, respectively. In one, the NORs were located in both homologues of chromosomes 6 (accession H197; Figure 2), and in the other in chromosome 3 and the same position on chromosome 6 (accession H340; Figure 2). The HKL of standard individuals was $38.34 \pm 1.24 \mu\text{m}$ with a 1C DNA content of $6.27 \pm 0.083 \text{ pg}$ (H274; Table 2). The HKL of the NOR translocation heterozygote H340 was slightly lower and the genome size

slightly smaller ($30.03 \pm 1.99 \mu\text{m}$ and $6.07 \pm 0.031 \text{ pg}$; Table 2) while NOR translocation homozygote H197 had HKL of $34.97 \pm 3.98 \mu\text{m}$ and genome size of $6.05 \pm 0.011 \text{ pg}$ (Table 2).

Cytotype B⁵B⁵ (2n = 2x = 10)

In the six B⁵B⁵ individuals, the karyotype comprised two sub-metacentrics (chromosomes 2 and 5), one sub-telocentric (chromosome 4) (again reflecting homoeologies with B⁷ and A genomes), a large sub-metacentric fission/fusion chromosome F²(6–7), and a sub-metacentric fission/fusion chromosome F³(1–3) (Figure 2). In B⁵B⁵, the HKL was $29.67 \pm 2.58 \mu\text{m}$ and the 1C DNA amount $4.86 \pm 0.002 \text{ pg}$ (Table 2). It has been proposed previously that B⁵B⁵ results from two fusions, one identical to that in the B⁶B⁶ karyotype (F¹ = F²). The second fusion (F³) was more complex, but has been interpreted to be a result of chromosome 1 and 3 fusion (Additional file 1: Figure S1) relocating the NOR to the short arm of an enlarged fusion chromosome F³ (Additional file 1: Figure S1).

5S and 35S rDNA localisation

The three species of *Prospero* and all cytotypes invariably had one 35S rDNA locus per genome (Figure 2, Additional file 2: Figure S2). Its chromosomal localization was predominantly interstitial and adjacent to the centromere, except in *P. hanburyi* where it was sub-terminal. Either one or two 5S rDNA loci were found with a third, minor, locus in *P. obtusifolium* (Figure 2, Additional file 2: Figure S2). Locations of these loci were more variable than the 35S rDNA loci.

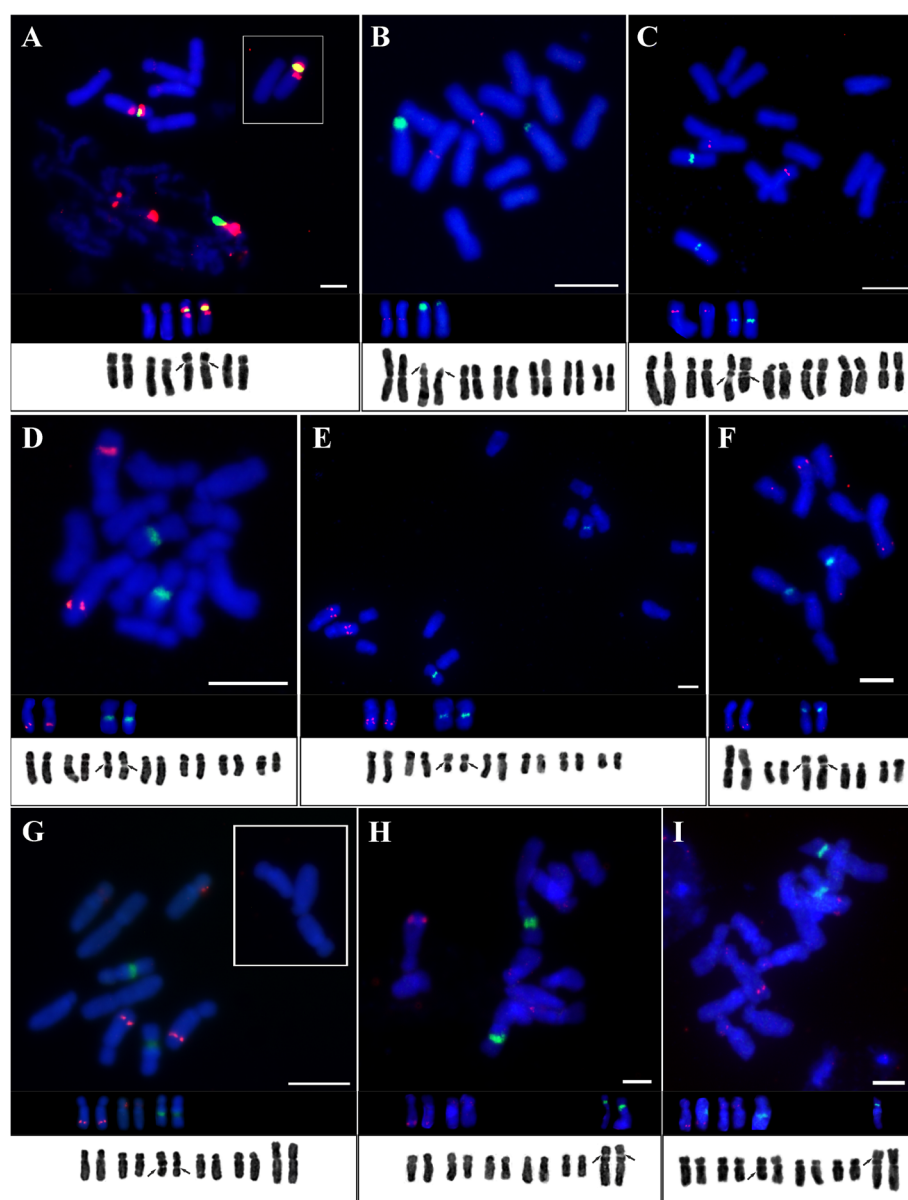


Figure 2 Karyotypes and localization of 35S (green) and 5S (red) rDNA loci in diploids of *Prospero*. (A) *P. obtusifolium*; (B) *P. hanburyi*; (C-I) *P. autumnale* complex: (C) AA; (D) B⁷B⁷; (E) B⁷B⁷ with duplicated 5S rDNA locus in chromosome 1; (F) B⁵B⁵; (G) B⁶B⁶; (H) B⁶B⁶ with homozygous NOR translocation (NOR in pair of chromosome 6); (I) B⁶B⁶ with heterozygous NOR translocation (NOR in one of each chromosome 3 and 6). Insets in (A) and (G) show chromosomes of a single cell which were lying at some distance from the main chromosome group and either could not be photographed together using high magnification objectives or were too far apart to clearly demonstrate chromosome morphology while showing the whole field. Scale bar = 5 μ m.

- i. The 35S rDNA locus of *P. hanburyi* was sub-terminal on the short arm of chromosome 2, whereas the single 5S rDNA locus was located on the long arm of metacentric chromosome 1 adjacent to the centromere (Figure 2B).
- ii. In *P. obtusifolium*, the 35S rDNA locus was on chromosome 3, flanked on each side by a 5S rDNA locus (Figure 2A). An additional minor 5S rDNA locus was seen occasionally, located on the long arm of chromosome 2 (Figure 2A).
- iii. All cytotypes of *P. autumnale* possessed a single interstitial 35S rDNA locus, usually closely adjacent to a centromere. There were either one or two 5S rDNA loci in different cytotypes (Figure 2):
 - in the AA cytotype, a single 5S rDNA locus was found in the pericentric region of the short arm

Table 2 Genome size, karyotype length and rDNA loci number and localization in *Prospero*

Cytotype (Accession number)	5S and 35S rDNA loci number and localization ¹ , 2	Genome size 1C (pg) ± SD	Chromosome size HKL (µm) ± SD
<i>Prospero hanburyi</i> (H397)	5S (L-P ^{chr1}) 35S (S-ST ^{chr2}) 	6.81 ± 0.017	44.90 ± 4.04
<i>P. obtusifolium</i> (H540)	5S (L-P ^{chr2} , L-P ^{chr3} , S-P ^{chr3}) 35S (S-P ^{chr3}) 	4.94 ± 0.039	29.01 ± 0.77
<i>P. autumnale</i> complex AA (H551)	5S (S-P ^{chr2}) 35S (L-P ^{chr3}) 	7.85 ± 0.045	48.35 ± 7.15
B ⁷ B ⁷ (H450)	5S (L-D ^{chr1}) 35S (L-P ^{chr3}) 	4.23 ± 0.048	33.76 ± 1.45
(H424)	5S (L-D ^{chr1}) 35S (L-P ^{chr3}) 	4.45 ± 0.023	28.70 ± 1.74

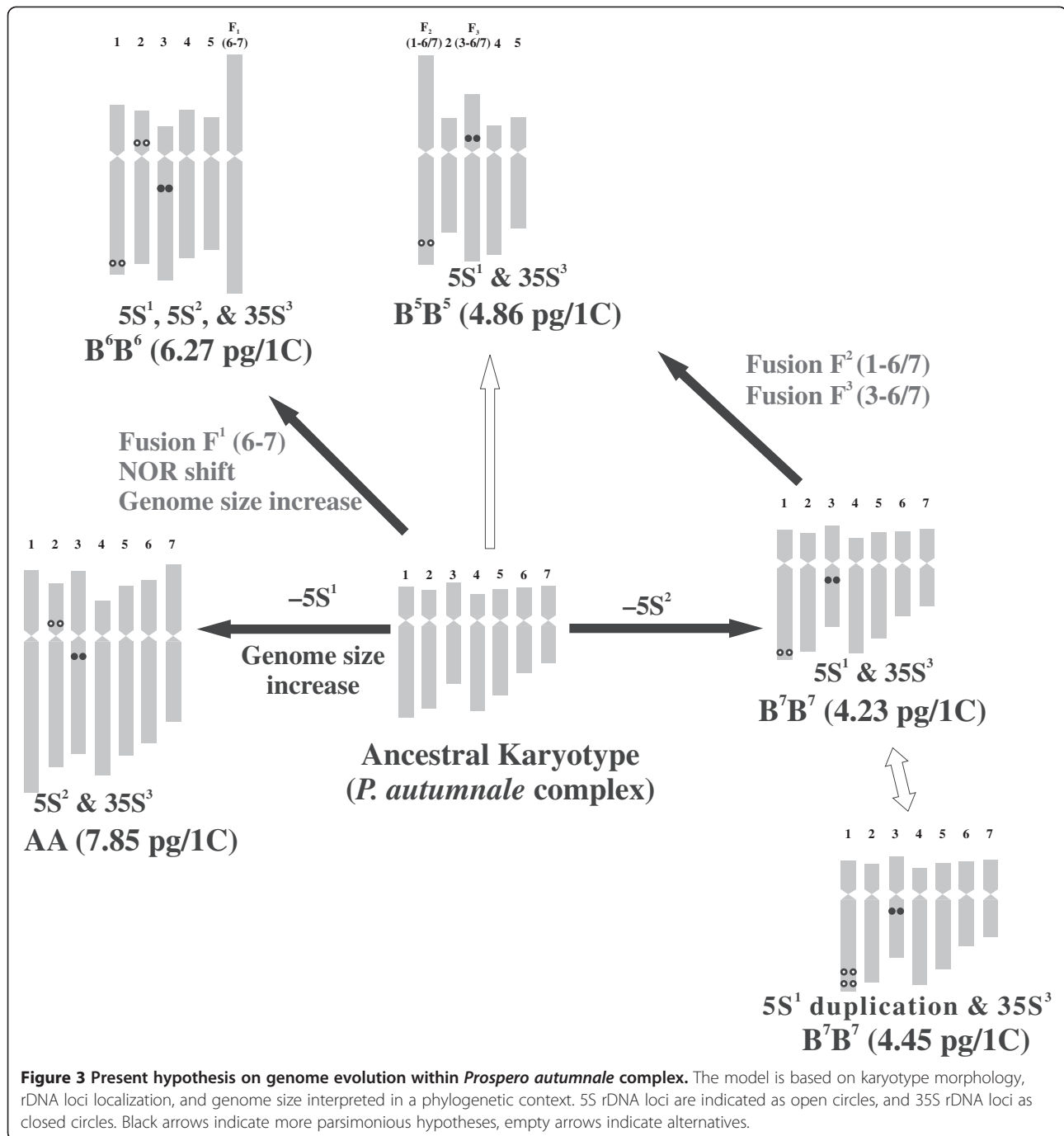
Table 2 Genome size, karyotype length and rDNA loci number and localization in *Prospero* (Continued)

B ⁶ B ⁶ (H274)	5S (L-D ^{chr1} , S-P ^{chr2}) 35S (L-P ^{chr3})		6.27 ± 0.083	38.34 ± 1.24
B ⁵ B ⁵ (H640)	5S (L-D ^{chr1}) 35S (S-P ^{chr3})		4.86 ± 0.002	29.67 ± 2.58
Translocations B ⁶ B ⁶ (H197)	5S (L-D ^{chr1} , S-P ^{chr2}) 35S (S-P ^{chr6})		6.05 ± 0.011	34.97 ± 3.98
B ⁶ B ⁶ (H340) ³	5S (L-D ^{chr1} , S-P ^{chr2}) 35S (L-P ^{chr3} , S-P ^{chr6})		6.07 ± 0.031	30.03 ± 1.99

¹ L, long arm; S, short arm; D, distal (interstitial) location of 5S rDNA; P, pericentric location of 5S or 35S rDNA; ST, subterminal location of 35S rDNA; chr, number of the chromosome.
²Scale bar = 1 μm; only the chromosomes bearing rDNA are shown: filled and open circles indicate position of 35S and 5S rDNA loci, respectively.
³Heterozygote; both chromosomes carrying 35S rDNA (chromosome 3 and chromosome 6) are shown.

of chromosome 2 (Figure 2C). The 35S rDNA locus was close to the centromere in the long arm of chromosome 3;
– cytotype B⁷B⁷ usually had a single 5S rDNA locus localized interstitially within distal region of the

long arm of chromosome 1. Some individuals, however, had two loci in close proximity at this position, suggesting either local duplication of this chromosomal region or of the locus itself (Figure 2D–E). The single 35S rDNA locus was



- pericentromeric on chromosome 3 long arm (Figure 2D–E), in a similar location to that in AA (Figure 2C);
- cytotype B⁶B⁶ always had two 5S rDNA loci, a smaller one in the pericentric region of the short arm of chromosome 2 as in cytotype AA (Figure 2G) and a larger one in the distal region of the long arm of chromosome 1 as in B⁷B⁷. In most

plants, there was a single 35S rDNA locus interstitial in the long arm of chromosome 3, although further from the centromere than that in AA and B⁷B⁷ (Figure 2C–E). Two plants differed from the standard pattern in their rDNA localization. In one individual, the 35S rDNA locus was close to the centromere in the short arm of submetacentric chromosome 6 (Figure 2H). In the

other, one copy of the locus was detected in chromosome 6 and the other in the typical position on chromosomes 3 (Figure 2I).

- In the B⁵B⁵ cytotype, putative fusion chromosome F²(6–7) (Additional file 1: Figure S1) [26] had a 5S rDNA locus distal in one arm (Figure 2F). The 35S rDNA locus was localized interstitially close to the centromere within the short arm of the second-largest chromosome in the complement (Figure 2F), the putative fusion chromosome F³(1–3) (Additional file 1: Figure S1) [26].

Phylogenetic relationships within *Prospero* based on ITS sequence data

Sequence analyses of ITS1 and ITS2 regions, including the intervening 5.8S coding region, of 35S rRNA have provided insights into the relationships amongst the diploids of *Prospero* (Figure 4). The length of the ITS region of the 49 analyzed diploid *Prospero* accessions ranged from 778 to 785 bp and the final, aligned dataset was 793 bp long. The maximum parsimony analysis of the ITS dataset resulted in four most parsimonious trees with a length of 216 steps (65 parsimony informative characters, consistency index [CI] = 0.926, retention index [RI] = 0.965, rescaled consistency index [RC] = 0.893). The final tree was rooted with two outgroup taxa (*Othocallis siberica* and *Dipcadi* sp., both in family Hyacinthaceae; Table 1). The genus *Prospero* was monophyletic (BS 99; Figure 4B). *P. obtusifolium* (6 individuals) and *P. hanburyi* (3 individuals) each formed well-supported clade (bootstrap support, BS 100). *P. obtusifolium* and *P. hanburyi* ITS regions differed by 29 substitutions, one of which is within one of two insertions (3 and 4 bp long) shared only by these two taxa (Additional file 3: Figure S3). *P. hanburyi* had an additional unique insertion of 3 bp. *P. hanburyi* and *P. obtusifolium* differed from B⁷B⁷ diploids by the above mentioned two shared insertions and by 13 and 28 substitutions, respectively (Additional file 3: Figure S3). *P. obtusifolium* (BS 100) was recovered as sister to clade comprising *P. hanburyi* and *P. autumnale* (BS 81). The *P. autumnale* complex formed a monophyletic and well-supported clade (BS 90). Within this clade cytotype AA (six individuals), formed a monophyletic sub-clade (BS 100; Figure 4). ITS sequences of all AA individuals were identical. ITS region of cytotype AA has two unique insertions (1 and 2 bp long, respectively; Additional file 3: Figure S3). The B⁷B⁷ cytotype (17 individuals) forms a well-supported clade (BS 98; Figure 4). This was the only cytotype within which ITS sequence variation has been observed (four distinct B⁷B⁷ groups, each having a unique substitution; Additional file 3: Figure S3). Interestingly, B⁷B⁷ clade

includes all six individuals of the B⁵B⁵ cytotype nested within it (Figure 4A), or forming a sub-clade of unresolved relationship to B⁷B⁷ subclade with a bootstrap support of 86 (Figure 4B). All B⁵B⁵ individuals shared a unique 2 bp insertion compared with the B⁷ ITS sequence.

The B⁶B⁶ cytotype ($2n = 2x = 12$; eleven individuals) formed a well-supported monophyletic group (BS 100; Figure 4). The two B⁶B⁶ individuals with the 35S rDNA translocation did not show any ITS variation compared to other analyzed individuals. The ITS sequences of genomes B⁶ shared four unique substitutions (Additional file 3: Figure S3).

Discussion

Chromosome numbers and karyotype variation

The genus *Prospero* is highly variable in chromosome number and chromosome structure. Basic numbers have changed by dysploidy ($x = 4, 5, 6$, and 7) and, superimposed on this, high levels of auto- and allopolyploidy have evolved [24,26]. Three species are commonly recognized in the genus: *P. obtusifolium*, confined to the western Mediterranean islands and adjacent mainland, exclusively diploid with $2n = 8$; *P. hanburyi* from the Levant, also a diploid but with $2n = 14$; and the widespread *P. autumnale* complex with basic numbers of $x = 5, 6$, and 7 and an elaborate, reticulating auto- and allopolyploid series (from $3x$ to about $20x$, but most frequently $4x$ and $6x$; [24-26,28,29,34,37,41]).

Within the *P. autumnale* complex, four distinct cytotypes have been described and characterized so far [24,26]. A fifth genome, designated as B^{7*} (or C), with chromosomes slightly smaller than B⁷ but of the same complement morphology, has so far been found only in allopolyploids on Crete [26]. The diploid cytotypes differ in chromosome number ($2n = 2x = 10$ [B⁵B⁵], 12 [B⁶B⁶], 14 [AA, B⁷B⁷]), in karyotype structure with one and two putative fusions resulting in B⁶ (F¹) and B⁵ (F² and F³) respectively, in NOR position, and in genome size, with a major difference in DNA amount between the A genome and the other three [26,37]. These studies are supported here, except that a few individuals with translocations were detected.

A combination of karyotypic features (chromosome size, morphology, NOR position, unique and stable locations of 5S and 35S rDNA loci, genome size) allows unambiguous identification of each cytotype as well as identification of homoeologous chromosomes between them (Figure 3). The karyotypes of *P. hanburyi* and *P. obtusifolium* differ from those of the *P. autumnale* complex to such an extent that it is impossible to infer any homoeologies between these taxa.

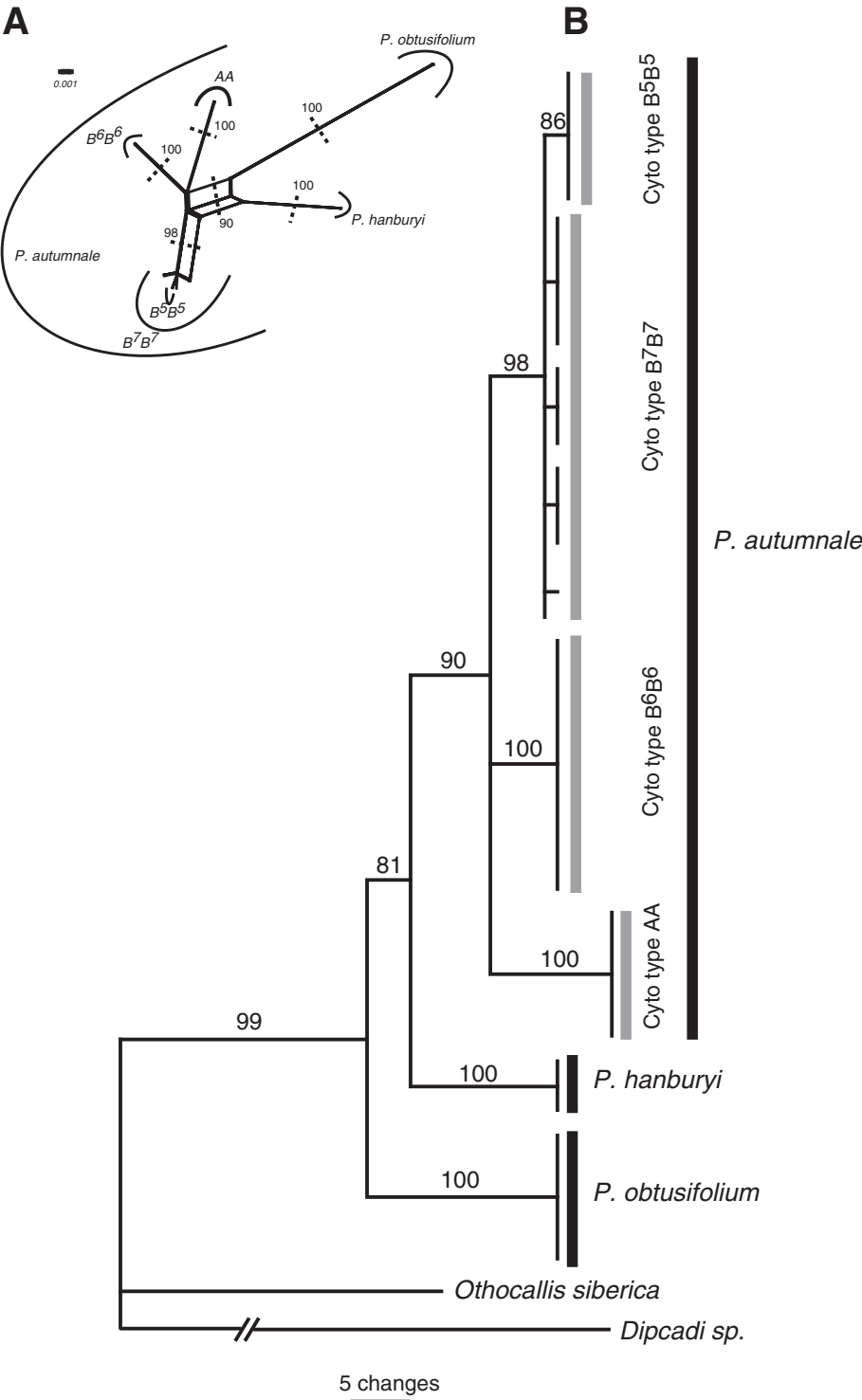


Figure 4 Phylogenetic relationships within the genus *Prospero* inferred from ITS sequence data. (A) NeighbourNet; (B) maximum parsimony phylogram.

Evolution of 5S and 35S rDNA loci

5S and 35S rDNA have been mapped to the chromosomes of all diploid species and cytotypes of the genus *Prospero*. This has allowed us to identify two and sometimes three chromosome pairs unambiguously (Figure 2). Thus, despite the high frequency of chromosomal rearrangements within and between populations of the *P. autumnale* complex - inversions, supernumerary segments, translocations, B-chromosomes [24,25,37,42] - all diploid cytotypes possess unique and stable locations of their rDNA loci (except for two B⁶B⁶ plants in this study with translocations). By contrast 5S rDNA proved to be more variable in locus number and location (Figure 2), a phenomenon observed in other plant groups [5,15,16,43,44]. Despite this variability, 5S rDNA is frequently more stable in its position than 35S rDNA, which may vary substantially in distribution between related species (e.g., in *Aloe* [45]) and even between cells in individuals of species of genus *Allium* [46].

P. obtusifolium exhibits a remarkable pattern of rDNA distribution, unique within *Prospero*: juxtaposition of a centromere and the 35S rDNA locus, with a 5S rDNA locus on each side (Figure 2A). Co-location of 35S and 5S rDNA within the same chromosome or chromosomal arm has, however, been reported in other plant groups [16,47-49], sometimes even as 5S rDNA units inserted within 35S rDNA repeats [17].

Within Hyacinthaceae, genera related to *Prospero* possess basic numbers of $x=7$ or higher [28]. *Prospero obtusifolium* forms the basal clade in the ITS-derived phylogeny. It probably represents an old segregate within the genus, which is estimated to be 6.43 Ma old [40], and has experienced chromosomal rearrangements leading to a drastic chromosome number reduction to $x=4$.

P. hanburyi is the only species in the genus to possess a subterminally localized 35S rDNA locus, instead of interstitial secondary constrictions adjacent to centromeres. It has been argued that a subterminal position for 35S rDNA is ancestral [50], but in *Prospero* it might also be associated with a high potential of 35S rDNA for generating chromosomal translocations [51,52]. The single 5S rDNA locus is located in unique chromosomal position close to the centromere of chromosome 1. It shares a common ancestry with the 5S rDNA locus of chromosome 1 in *P. autumnale*, as indicated by the phylogenetic analyses of the non-transcribed spacer region (K. Emadzade, H. Weiss-Schneeweiss et al., unpublished observations).

In contrast to the other two species, the diploids of the *P. autumnale* complex lend themselves to comparative karyotype analysis, due to the well-preserved chromosomal homoeology during evolution. Homoeology was first demonstrated in A and B⁷ diploid homoploid hybrids [35,36], and was extended to B⁷, B⁶ and B⁵ by analyses of meiotic pairing in diploid hybrids ([26,34], discussed

below). The position of 35S rDNA is relatively conserved in the complex: within the long arm of chromosome 3, except when affected by the fusion in cytotype B⁵B⁵. The NOR chromosome (3) in the B⁶ genome has a similar size and arm ratio to chromosome 3 in B⁷, but it differs in the proximity of the NOR to the centromere, probably as a result of paracentric inversion ("NOR shift", [24]). This regularity of interstitial position of 35S rDNA supports the earlier hypothesis [51] that it might provide greater karyomorphological stability during race or species evolution. The 5S rDNA loci are either interstitial in the distal part of the long arm of submetacentric chromosome 1 and/or proximal in the short arm of submetacentric chromosome 2, except where fusion has occurred in cytotype B⁵B⁵ (Figures 2 and 3). The only variation observed in the complex was a putative duplication of 5S rDNA locus in some copies of chromosome 1 of B⁷B⁷. Although phylogenetic analyses of ITS sequences did not ascribe any evolutionary significance to this duplication, phylogenetic analyses of the more variable 5S rDNA spacer (K. Emadzade, H. Weiss-Schneeweiss et al., unpublished observations) indicated that individuals carrying this duplication are more closely related to each other than to individuals carrying a single copy of the locus.

In addition to the between-cytotype variation in the number and distribution of 5S rDNA loci, variation in FISH signal intensity has frequently been observed (e.g., in the B⁶B⁶ cytotype; Figure 2G). Signal strength differences are likely to be correlated with copy-number variation at the target site [53].

Phylogenetic interpretation of chromosomal variation in *Prospero*

The phylogeny of *Prospero*, inferred from ITS sequences, strongly supports monophyly of each species and diploid cytotype. *P. obtusifolium* and *P. hanburyi* are always recovered as subsequent sister groups to the *P. autumnale* complex. Neither species, however, has obvious chromosome homoeology with *P. autumnale*. By contrast, the ITS phylogeny coupled with knowledge of chromosome numbers, karyotype structure, and genome size allows us to test previous hypotheses concerning the direction and mechanisms of karyotype evolution within the *P. autumnale* complex (Figure 3, Additional file 1: Figure S1).

We offer a modified and more detailed model of the chromosomal changes involved in the origin of the cytotypes (Figure 3). Each cytotype forms a well-supported clade, with cytotype AA being the most distinctive. Cytotype AA is found only in the western distribution area of the genus, adjacent to the Atlantic Ocean and might have been isolated by a Pleistocene glacial advance.

The ITS phylogeny supports the origin of cytotype B⁵B⁵ from B⁷B⁷ rather than from B⁶B⁶, with genome B⁶ being sister to B⁷. The close relationship of the localized

cytotype B⁵B⁵ within the widespread cytotype B⁷B⁷ (Figure 4A) suggests its recent origin, and that it is the youngest segregate of the complex. Intra-cytotype ITS sequence variation has only been observed within the widespread cytotype B⁷B⁷. This contrasts with a lack of variation in all other more geographically localized or endemic cytotypes and species. Thus phylogenetic and chromosomal data, and particularly the distribution of 5S rDNA loci in cytotypes B⁵B⁵, B⁶B⁶, and B⁷B⁷, suggest independent and not sequential dysploidy: from $x = 7$ to $x = 6$ and independently from $x = 7$ to $x = 5$.

Model of karyotype evolution in *Prospero autumnale* complex

Genome size estimations presented in the current study (Table 2, Figure 3) differ from genome size measurements published previously (Additional file 1: Figure S1) [26]. These previous measurements have been performed using Feulgen densitometry which could account, at least partially, for the discrepancy. However, *Prospero* genome size measurements reported in another study [37] are largely congruent with our data.

The chromosome number and structure of the *Prospero* ancestral karyotype (genus-wide) remains obscure, as do the karyotype relationships of the three species (K. Emadzade, T.-S. Jang, H. Weiss-Schneeweiss et al., unpublished observations). The ancestral chromosome number of the *Prospero autumnale* complex has been inferred as $x = 7$, and this is also supported by phylogenetic reconstructions using extended plastid, ITS, and 5S rDNA spacer sequence datasets (K. Emadzade, H. Weiss-Schneeweiss et al., unpublished observations), with the ancestral karyotype similar in overall morphology to the A and B⁷ genomes [24,34] (Figure 3). These genomes each possess one 5S rDNA locus either in long arm of chromosome 1 (5S¹; B⁷) or in the short arm of chromosome 2 (5S²; A). Sequencing of the NTS regions of these loci shows them to be distinct (data not shown). We propose that the ancestral genome had both of these loci. The ancestral genome could have resembled A or B⁷ in size, or indeed be different from both, but increase is thought to be predominant to, and more rapid than, genome decrease. So resemblance of B⁷ to the ancestral karyotype is likely to be the most parsimonious, and genome increase might have occurred in the western refugium during a glacial maximum ([26], J. Parker, unpublished observations).

Loss of 5S rDNA from chromosome 1 (5S¹) of the ancestral karyotype has likely accompanied evolution of cytotype AA. Its evolution has also been accompanied by nearly 70% genome size increase (Figure 3). Loss of the 5S² rDNA locus from the ancestral karyotype would give rise to cytotype B⁷B⁷ (Figure 3), now widespread across the whole Mediterranean basin. Interestingly, seven of the seventeen B⁷ diploids analysed carried a duplication of the 5S¹ rDNA locus.

Genome B⁶ may have originated from the ancestral karyotype with $x = 7$ by fusion of chromosomes 6 and 7 (Figure 3). It is also necessary to postulate a pericentric inversion and loss of a centromere in its evolution [26]. Previously, it had been proposed that the B⁶ genome evolved by chromosome fusion directly from B⁷ (Additional file 1: Figure S1). Evidence for the direct evolution from an ancestral karyotype rather than directly from B⁷ comes from the retention of both the 5S rDNA loci by B⁶. The analysis of meiotic pairing in hybrids does not differentiate between the two hypotheses [24,26]. The genome of B⁶ is 44% larger than B⁷, and the difference affects all chromosomes of the complement nearly equally. This is observed as bivalent asymmetry during meiosis in hybrids.

It was also proposed that B⁵ arose directly from B⁶ by a second fusion event [24]. The evidence came from the gross similarity of the largest fusion chromosomes in genomes B⁶ and B⁷ (thus they proposed F¹ = F²), and the presence of two trivalents at meiosis in B⁵B⁷ hybrids. However, the molecular evidence presented here is consistent with B⁵ arising from B⁷, but supports evolution of B⁶ directly from an ancestral species of *P. autumnale*. The fusion chromosomes in B⁵ [F²(1-6/7), F³(3-6/7)] and B⁶ [F¹(6-7)], therefore, have independent origins. No molecular markers are yet available to unequivocally identify chromosome 6 and 7, so the relationships of the fusion chromosomes cannot be explored more closely. The fusion chromosome F² in cytotype B⁵ involving chromosome 1 and chromosome 6 or 7 [earlier proposed to be = to F¹(6-7)], gives rise to a near-metacentric, the largest in the complement. As expected, this carries a 5S rDNA locus, which has been confirmed as 5S¹ by sequencing (K. Emadzade, H. Weiss-Schneeweiss et al., unpublished observations). In addition, the genome size of B⁵ is 12% higher than B⁷ but it cannot be established at what point this may have occurred. The cytotype B⁵B⁵ is probably the most recently evolved diploid in the complex and is endemic to Libya, where it is the only race [34].

Conclusions

Phylogenetic analysis has confirmed fusion and basic number reduction as opposed to fission and basic number increase as the evolutionary mechanisms characterizing karyotype evolution in the *P. autumnale* complex. Dysploidy has occurred twice via independent fusions, once perhaps ancestral from $x = 7$ to $x = 6$, and later a second time from $x = 7$ to $x = 5$. This extensive chromosomal evolution contrasts very strongly with a lack of morphological diagnostic features within the genus, which are particularly weak within the *P. autumnale* complex [29,33,54]. New species described in last few decades usually refer to small populations that differ mostly in quantitative characters, whose evolutionary

significance needs to be evaluated using a more thorough sampling. Diversification and evolution of this genus, then, has occurred primarily through genome restructuring, with little involvement of morphological change. Genetic processes may, of course, be implicated in the generation of chromosomal change. Thus, the genus *Prospero*, and in particular the *P. autumnale* complex, provides a model system for studying the role of chromosomes in plant diversification.

This study of diploids in *Prospero* has laid foundations (1) to address the evolution of auto- and allopolyploidy within the complex which appear to follow different evolutionary trajectories; ([26,42], H. Weiss-Schneeweiss et al., unpublished observations), (2) to interpret the mechanisms involved in the origin and persistence of the many other types of chromosomal rearrangements that are found abundantly across the complex (such as B-chromosomes of many types, supernumerary segments on several chromosomes, translocations, and para- and pericentric inversions), and (3) to investigate the patterns of evolution of repetitive DNAs within the genus.

Methods

Plant material

Bulbs of all three *Prospero* species were collected from natural populations across the range (Table 1, Figure 1) and grown in the Botanical Garden of the University of Vienna. Due to high level of chromosomal variation, all individual bulbs were karyotyped prior to the FISH and phylogenetic analyses to select diploids (603 bulbs in total; T.-S. Jang, H. Weiss-Schneeweiss, unpublished observations). Where possible at least five bulbs with a "standard" (most common, without structural polymorphisms) karyotype were selected for the analyses; only three individuals with healthy root tips were available in *P. hanburyi*. *Othocallis siberica* and *Dipcadi* sp. (both in family Hyacinthaceae) were used as outgroup in phylogenetic analyses.

Karyotype analysis and fluorescence *in situ* hybridization (FISH)

Actively growing root-tip meristems were pretreated with 0.05% aqueous solution of colchicine for 4 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.

Chromosome counting and basic karyotype analyses were performed using the standard Feulgen staining technique [55]. Ideograms (Additional file 2: Figure S2) were constructed based on measurements of at least five well-spread metaphase plates per individual (not shown) and measurements were used to calculate Haploid Karyotype Length (HKL). A single ideogram of each species and cytotype is provided, except for cytotypes B⁷B⁷ and B⁶B⁶

in which structural chromosomal variants were found (Table 2). Idiograms were constructed using Autoidiogram software (courtesy of Dr Wolfgang Harand, formerly University of Vienna; for details see [55]).

Chromosomal spreads for FISH were prepared by enzymatic digestion and squashing, as described earlier [4,16] with some modifications. Briefly, material was digested with 1% cellulase Onozuka (Serva, Heidelberg, Germany), 1% cytohelicase (Sigma-Aldrich, Vienna, Austria), and 1% pectolyase (Sigma-Aldrich) for 18 min at 37°C. Cover slips were removed at -80°C and preparations air-dried. FISH followed the established protocol [16,56]. Probes used for FISH were: 35S (18S/25S) rDNA from *Arabidopsis thaliana* in plasmid pSK+; 5S rRNA genic region from *Melampodium montanum* in plasmid pGEM-T Easy. Probes were labeled with biotin or digoxigenin (Roche, Vienna, Austria) either directly by PCR (5S rDNA) or using a nick translation kit (35S rDNA; Roche, Vienna, Austria). Digoxigenin was detected with antidigoxigenin antibody conjugated with FITC (5 µg mL⁻¹; Roche, Vienna, Austria) and biotin with ExtrAvidin conjugated with Cy3 (2 µg mL⁻¹; Sigma-Aldrich, Vienna, Austria). Preparations were analyzed with an AxioImager M2 epifluorescent microscope (Carl Zeiss, Vienna, Austria), images captured with a CCD camera, and processed using AxioVision ver. 4.8 (Carl Zeiss, Vienna, Austria) with only those functions that apply equally to the whole image. For rDNA localization, a minimum of 20 well-spread metaphases and prometaphases was analysed for each individual.

DNA amplification, sequencing, and phylogenetic approach

Total genomic DNA was extracted from silica-dried leaf material using the standard CTAB procedure [57] with some modifications [58]. The nuclear ITS region (partial 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and partial 25S rRNA gene) was amplified with universal primers (ITS 18 s F and ITS 26 s R, [59]).

Polymerase chain reactions were carried out using 0.4 mM of each primer, ReddyMix (Abgene, Vienna, Austria) including 2.5 mM MgCl₂ and 4% (v/v) dimethyl sulfoxide (DMSO). All PCR reactions were performed on an ABI thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA) with the initial 3 min at 95°C, followed by 30 cycles each of 30 s at 96°C, 30 s at 58°C, and 2 min at 72°C, followed by a final elongation at 72°C for 8 min. Amplified fragments were checked on 1% (w/v) agarose gel and purified using exonuclease I (ExoI) and calf intestine alkaline phosphatase (CIAP) according to the manufacturer's protocol (Fermentas, St. Leon-Rot, Germany). The purified fragments were directly sequenced using the PCR primers and dye terminator chemistry following the manufacturer's protocol (Applied Biosystems). Sequencing

reactions were run on a 48-capillary sequencer (3730 DNA Analyzer, Life Technologies). Sequences were assembled in SeqManII (Lasergene, Madison, WI) and manually aligned in BioEdit software ver. 7.0.5.3 [60]. Indels were coded as binary characters following the “modified complex coding method” [61] using the program SeqState version 1.36 [62], and the dataset with coded gaps was used for all analyses. A heuristic search for most parsimonious (MP) trees was performed using PAUP 4.0.b10 [63]. The analyses involved 1000 replicates of random sequence addition, with tree bisection–reconnection (TBR) and branch swapping, saving no more than 10 trees per replicate. All characters were equally weighted and treated as unordered. Strict consensus trees were computed from all equally most parsimonious trees. Internal branch support was estimated using non-parametric bootstrapping [64] with 10 000 replicates and 10 addition sequences replicates. Neighbor Net implemented in SplitsTree4 v. 4.11.3 [65], with gaps and ambiguous sites treated as missing data, was used to create the ITS network. Split support was calculated with 1000 bootstrap replicates. All ITS sequences are deposited in GenBank (accession numbers provided in Table 1) and the alignment and phylogeny are deposited in treeBASE (submission number 14243).

Genome size estimation by flow cytometry (FCM)

The 1C values of all *Prospero* species and each cytotype of *P. autumnale* complex were measured using FCM with *Solanum pseudocapsicum* (1C = 1.29 pg, [66]) as the internal standard. Approximately 25 mg fresh leaves from each plant sample were co-chopped together [67] with standard material in Otto's buffer I [68], and filtered through a 30 µm Nylon mesh. After 30 min RNase treatment at 37°C, the nuclei were stained in Otto's buffer II [68] containing propidium iodide as the DNA stain. A CyFlow ML flow cytometer equipped with green laser (100 mW, 532 nm; Cobolt Samba; Cobolt AB, Stockholm, Sweden) was used for genome size estimation. The 1C values were calculated according to previously published formula [66].

Availability of supporting data

Nucleotide sequences are available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) under numbers KC899267–KC899317. Nucleotide alignment and phylogenetic analyses are deposited in treeBASE under study 14243 (<http://purl.org/phylo/treebase/phyloids/study/TB2:S14243>).

Additional files

Additional file 1: Figure S1. Previous hypothesis on karyotype evolution in the *Prospero autumnale* complex [26]. Black arrows indicate more parsimonious hypotheses, empty arrows indicate alternatives.

Additional file 2: Figure S2. Ideograms of each of the standard (most frequent and without polymorphisms) diploid species and cytotypes analysed.

Additional file 3: Figure S3. Alignment of variable nucleotide positions in the analysed ITS region.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TS-J carried out the cytogenetic studies, participated in the sequence alignment and drafted the manuscript. KE carried out sequencing of the ITS regions, sequence alignments, and phylogenetic analyses, and helped to draft the manuscript. JP provided plant material, participated in the design of the study and data interpretation, and helped to draft the manuscript. EMT carried out genome size measurements. ARL participated in the design of the study and data interpretation, and helped to draft the manuscript. FS provided plant material and helped to draft the manuscript. HW-S conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Author details

¹Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030, Vienna, Austria. ²Cambridge University Botanic Garden, Cambridge CB2 1JF, UK. ³Queen Mary College, University of London, London, UK. ⁴Dornacher Strasse 1, Linz 4040, Austria.

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Expansion of tandem repeat *PaB6* coincides with chromosomal rearrangements in the chromosomally hyper-variable *Prospero autumnale* complex (Hyacinthaceae)

Tae-Soo Jang^{1,5}, Khatere Emadzade^{1,5}, Jiří Macas², Petr Novák², Ales Kovařík³, John Parker⁴, Hanna Weiss-Schneeweiss¹

¹Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria;

²Czech Academy of Sciences, Institute of Plant Molecular Biology, Ceske Budejovice, Czech Republic;

³Czech Academy of Sciences, Institute of Biophysics, Brno, Czech Republic;

⁴Cambridge University Botanic Garden, Cambridge, CB2 1JF, UK

⁵these authors contributed equally to this study

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Abstract

New satellite DNA *PaB6* has been isolated and characterized in genus *Prospero* among repetitive DNA clusters identified from analyses of NGS of repetitive genome fraction of one of the cytotypes. Analyses of 249 bp long *PaB6* monomer revealed its higher order structure, presence of several intact and truncated telomeric repeats, and high methylation levels. Whereas *PaB6* originated in ancestors of the genus *Prospero*, it underwent significant amplification only in one of the three species of the genus, chromosomally variable *P. autumnale* complex. This Mediterranean taxon consists of four basic diploid cytotypes, each having unique combination of chromosome number ($x = 5, 6$, and 7), genome size, and rDNA loci number and distribution. Each of the diploid cytotypes possessed distinct copy number of *PaB6* detected in pericentric regions of several to all chromosomes. Structured variation in copy number was encountered only in two lineages of cytotype B^7B^7 ($x = 7$). This variation correlated with the presence of single or duplicated locus of 5S rDNA in chromosome 1 of cytotype B^7B^7 , with single locus (ancestral condition) being associated with higher copy number of *PaB6* detected in all chromosomes of the complement. Highest *PaB6* copy numbers were detected in two cytotypes, B^6B^6 and B^5B^5 , which underwent independent chromosomal fusions from $x = 7$ leading to dysploid chromosome number change ($x = 6$ and $x = 5$, respectively). Thus, independent amplification of satDNA *PaB6* coincides with and might result from elevated rates of chromosomal rearrangements in these two evolutionary well-defined lineages. First generation diploid homoploid hybrids of three genomic combinations all displayed additive patterns of *PaB6* distribution in chromosomes inherited from parental genomes. Mechanisms of *PaB6* amplification and its potential association with centromeric regions are discussed.

Keywords amplification · chromosomal evolution · copy number · fluorescence *in situ* hybridization (FISH) · pericentric satellite DNA · *Prospero* · tandem repeats

Abbreviations

CIAP: Calf intestine alkaline phosphatase
DAPI: 4',6-Diamidino-2-phenylindole
eccDNA: Extrachromosomal circular DNA
ExoI: Exonuclease I
FISH: Fluorescence *in situ* hybridization
NGS: Next-generation sequencing
rDNA: Ribosomal DNA
satDNA: Satellite DNA
SSC: Saline-sodium citrate buffer
SDS: Sodium dodecylsulfate

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 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 and consists of long arrays of late-replicating, tandemly arranged, head-to-tail repeats (Charlesworth et al. 1994; Richard et al. 2008). Many satDNA monomers are 160/180 bp or 320/370 bp long (Hemleben 1993; Jiang et al. 2003; Hemleben et al. 2007), but other lengths have also been found, particularly in monocots (Vershinin et al. 1994, 1995; Grebenstein et al. 1996; Ambrožová et al. 2011) but also in dicots (e.g. Kolchinsky and Gresshoff 1995; Matyášek et al. 1997; Macas et al. 2007, 2011).

Satellite DNA is transcriptionally inactive and subject to methylation, histone modification, and chromatin remodelling (Volkov et al. 2006; Hemleben et al. 2007). It is preferentially localized in heterochromatic pericentromeric and subtelomeric chromosomal regions, but also interstitially (Charlesworth et al. 1994; Hemleben et al. 2007). No general function has been ascribed to satDNA (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 (Pezer 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; Pezer et al. 2012).

Higher plant genomes have few-to-many families of satDNAs (Hemleben et al. 2007; Macas et al. 2007, 2011). Individual satDNA 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). Groups of related taxa were proposed to share a common “library” of satellite DNA families each of which might follow own evolutionary trajectories (Meštrovič 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 (Meštrovič et al. 1998; Nijman and Lenstra 2001; Pons et al. 2004). Newly arising variants of a satDNA can rapidly replace previous copies due to concerted evolution, which results in intraspecific sequence homogenization (Plohl 2010). The efficiency of homogenization is satDNA-specific and depends on 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).

Several hypotheses have been proposed to explain the origin, diversity and evolution of satDNA families. Thus they may derive from fragments of standard components of the genome, such as 35S rDNA (Lim et al. 2004; Almeida et al. 2012) or 5S rDNA (Vittorazzi et al. 2011), or from transposable elements (Sharma et al. 2013). Processes such as replication slippage, unequal crossing-over, gene conversion, rolling circle replication, and 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) have been implicated in the origin, and subsequent evolution, of new monomers (Cohen and Segal 2009).

Recent developments in sequencing technology (high throughput next generation sequencing, NGS; Margulies et al. 2005) allow rapid and accurate generation of millions of random sequence reads from virtually any genome (Deschamps and Campbell 2010) and thus rapid identification of satDNAs (Macas et al. 2007). 454 pyrosequencing is particularly useful for identification and characterization of large repeat units, also satDNAs, as it generates long reads and so facilitates reconstruction of tandemly organized units (Macas et

al. 2007; Wheeler et al. 2008). NGS has enabled identification of novel satDNAs in a range of plant species (Macas et al. 2007, 2010, 2011; Swaminathan et al. 2007; Wicker et al. 2009; Renny-Byfield et al. 2011, 2012; Čížková et al. 2013).

The genus *Prospero* (L.) Speta (Hyacinthaceae) consists of two chromosomally and morphologically stable species *P. hanburyi*, $2n = 14$ and *P. obtusifolium*, $2n = 8$, and a chromosomally variable species-complex of *P. autumnale*. *P. autumnale* consists of a spectacular, and unparalleled, array of genetically, chromosomally, and phylogenetically well-defined, recently evolved, diploid cytotypes and their polyploid derivatives (Vaughan et al. 1997; Jang et al. 2013). This complex, which shows near-homogeneity in its morphology, 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 in *P. autumnale*, 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 westernmost Mediterranean and the Atlantic coast of Morocco, Portugal and Spain. The other two cytotypes, with $2n = 12$ (B^6B^6) and $2n = 10$ (B^5B^5), originated from a putative ancestor with $2n = 14$ via independent chromosome fusions. Cytotype B^6B^6 is endemic to Crete and B^5B^5 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. Among 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^6B^6 has been shown to be accompanied by independent genome size increases (Jang et al. 2013).

The current study involves comparative evolutionary analysis of a satellite *PaB6* identified by NGS from cytotype B^6B^6 . 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 homoploid diploid hybrids; (2) assess intra- and interspecific variation of reconstructed *PaB6* monomer at all levels of its organization - its DNA sequence, chromosomal localization, and genomic abundance; (3) analyze, in a phylogenetic context, the evolutionary trajectories of *PaB6* in all six diploid cytotypes of *P. autumnale* and their

diploid homoploid hybrids; (4) correlate the dynamics of *PaB6* evolution with 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 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 homoploid diploid hybrids (Table S1) using a modified C-TAB method (Doyle and Doyle 1987; Jang et al. 2013).

454 sequencing and clustering-based repeat identification

Cytotype B⁶B⁶ of *P. autumnale* was chosen for 454 pyrosequencing of the repetitive DNA fraction, as it has large blocks of heterochromatin (C-bands; Ebert et al. 1996). Sequencing of randomly sheared total genomic DNA was performed by 454 Life Sciences (Center for Medical Research, Graz, Austria) using a 454 GS FLX instrument with Titanium reagents (Roche Diagnostics). Sequencing half a 70×75 picotiter plate (corresponding to 1.8% coverage of the genome; Weiss-Schneeweiss, Macas et al. unpublished data) yielded 555 480 reads of average length 350bp. Quality-filtered reads (397 694) were subjected to graph-based clustering analysis, as described by Novák et al. (2010), to identify groups of reads representing repetitive elements (Weiss-Schneeweiss, Macas et al. unpublished data). 195 out of total 19 751 clusters, corresponding to the most abundant families of genomic repeats, were analyzed for their similarity to known sequences using RepeatMasker and BLAST searches against GenBank databases and a database of plant mobile element protein sequences (Neumann et al. 2011). 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 among all clusters as containing potential tandem repeats. Structural features of the tandem repeat motif and its subrepeats within the contigs of this cluster were further analyzed 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). Lengths of the *k*-mers ranged from 15 to 25 nucleotides, and the 25 bp-long *k*-mers were used for final sequence reconstruction. The consensus sequence is prepared for submitted to GenBank.

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'-ACCCTAATCAGAACTGGCCT; *PaB6R* 5'-TAGAGTTATTGGGATGTGTAC) facing outwards (Fig. 2a). These primers were used for amplification of reconstructed *PaB6* monomers from genomic DNA of diploid *Prospero* species and cytotypes and a few outgroup species (all of family Hyacinthaceae). PCR reactions consisted of 1×buffer (MBI Fermentas, Germany), 2.5 mM MgCl₂ (MBI Fermentas, Germany) 0.5 μM of each of the dNTPs (MBI Fermentas, Germany), 0.2 μM of each primer (Sigma Aldrich, Austria), and 1U of RedTaq polymerase (Sigma Aldrich, Austria). 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 sec at 94°C, 45 sec at 55°C, and 40 sec at 72°C followed by 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 satDNA *PaB6* were purified from the gel using Invisorb® Fragment clean up (Invitex, Germany). DNA was cloned using the pGEM-T Easy vector systems and JM109 competent cells (Promega, Madison, WI, USA) following 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). Amplified fragments were purified using exonuclease I (ExoI) and calf intestine alkaline phosphatase (CIAP) according to the manufacturer's protocol (Fermentas, St. Leon-Rot, Germany), cycle sequenced using big dye terminator chemistry (Applied Biosystems, Foster City, CA, USA)

and run on 48 capillary ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences of satDNA were manually aligned in BioEdit v.7.0.9 (Hall 1999). Phylogenetic analyses were performed using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) using the Kimura two-parameter substitution model implemented in MEGA v.4 software (Tamura et al. 2007). All sequences are prepared for submission to GenBank (Table S1).

Southern blot hybridization and slot blot

Abundance and restriction patterns of *PaB6* monomers in selected individuals were analyzed using the Southern blot technique. 1 µg of total genomic DNA of each *Prospero* species and cytotype was digested with 0.7 µl of *Bst*NI 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 B⁶B⁶ (clone 6 of individual H195). The probe was labelled either radioactively with ³²P (DekaLabel kit, MBI, Fermentas, Vilnius, Lithuania) or using a DIG-nick translation kit (Roche, Austria). Radioactively labelled probe was hybridized to the membrane and washed under high-stringency conditions, as described in Matyášek et al. (2011). Hybridization bands were visualized with a PhosphorImager (Storm, Molecular Dynamics, Sunnyvale, CA), and the data were processed in ImageQuant software (Molecular Dynamics; Sunnyvale, CA).

Hybridization of digoxigenin labelled probe (Dig Easy Hyb, Roche, Germany) to genomic DNA was carried out at 43°C for 14h, washed twice in 2×SSC (saline-sodium citrate buffer) containing 0.1% SDS (sodium dodecylsulfate) 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, Germany). Due to the lower sensitivity of chemiluminescent detection compared to radioactive systems, an additional hybridization experiment was performed with cytotypes B⁷B⁷ and AA, which had been shown to possess lower amounts of satellite DNA, using 1 µg and 3 µg of genomic DNA.

Copy number of *PaB6* in all species and cytotypes was estimated using the slot blot technique. Briefly, DNA concentration was estimated using Nanodrop 3300 (peqlab,

Germany) with PicoGreen (Invitrogen, country) as DNA stain. Two or three dilutions of genomic DNA (100 ng, 20 ng, and 2 ng for B⁶B⁶ and B⁵B⁵ cytotypes; 2000 ng, 200 ng and 20 ng for B⁷B⁷ and AA cytotypes; 2000 ng and 200 ng *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 0.75M NH₄OAc. Samples were blotted onto a positively charged Nylon membrane (peqlab, Germany) using 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, Germany).

Methylation levels

The methylation level of *PaB6* repeats in the B⁶ genome was assessed using a radioactive Southern blot (see above). The genomic DNA was digested with two restriction enzymes - *Bst*NI and *Scr*FI - which recognize and cut the same sequence, with *Scr*FI being methylation-sensitive.

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 B⁶ genome in plasmid pGEM-T Easy; genic region of 5S rDNA from *Melampodium montanum* (Asteraceae) in plasmid pGEM-T Easy, directly labeled with biotin or digoxigenin (Roche, Vienna, Austria) by PCR (Jang et al. 2013). Digoxigenin was detected with antidigoxigenin conjugated with FITC (5 µg mL⁻¹; Roche, Vienna, Austria) and biotin with ExtrAvidin conjugated with Cy3 (2 µg mL⁻¹; Sigma-Aldrich, Vienna, Austria), respectively.

Commercially available, directly Cy3-labeled, PNA probe to vertebrate telomeric sequences was used as the third probe, as described in the manufacturer's protocol (Telomere PNA FISH Kit/Cy3; Dako, Denmark). For the directly labeled PNA probe, after stringent washes in 2×SSC, 0.1×SSC and 2×SSC with 0.2% Tween20 at 42°C, 5 min each, preparations were mounted in antifade buffer Vectashield (Vector Laboratories, Peterborough, UK) containing DAPI counterstain (2 µg ml⁻¹) and stored at 4°C.

Preparations were analyzed with an AxioImager M2 epifluorescent microscope (Carl Zeiss, Vienna, Austria), images acquired with a CCD camera, and processed using AxioVision ver. 4.8 (Carl Zeiss, Vienna, Austria) with only those functions that apply equally to all pixels. At least 30 well-spread metaphases and prometaphases were analyzed in each individual.

Results

Satellite DNA identification and characterization of the monomers

Analysis of the NGS reads of repetitive DNA fraction of cytotype B⁶B⁶ ($2n = 12$) of *Prospero autumnale* resulted in 195 clusters (Weiss-Schneeweiss, Macas et al. unpublished data). The structure of cluster 9 (CL0009; Fig. S1), as well as its lack of similarity to any known sequence types deposited in GenBank and other databases, suggested that it represented satDNA. This cluster contained 8461 reads in 57 contigs, with total read length of 1333950 bp, estimated to represent 1.8% of the genome (the genome size of *P. autumnale* B⁶B⁶ 1C = 6.27 pg; Jang et al. 2013). Dot plot analyses of largest contigs of CL0009 confirmed a tandem repeat composition of the satellite (Fig. S1). 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). The mean GC content of the monomer was about 44%. This novel satellite DNA has been designated as *PaB6* - satellite DNA isolated from *P. autumnale* (*Pa*) cytotype B⁶B⁶ (*B6*).

Detailed analyses of *PaB6* monomer sequences, using the NGS dataset, revealed two large truncated subrepeats which have could have given rise to the present-day higher order monomer of 249 bp (Fig. 1b). Each of the two subrepeats is typically composed of three even smaller secondary subrepeats (Fig. 1c). The complex structure of this monomer is also indicated by the pattern of *PaB6* amplification using PCR (see below). Very few, and very small, inverted motifs were found within the monomer (Fig. 1c).

The monomer of *PaB6* contained seven intact vertebrate-type telomeric repeats (TTAGGG), usually dispersed among other sequences but in two instances forming dimers (Fig. 1a). Additionally, five imperfect telomeric-like repeats have been identified, and potential other repeats degenerated to a higher degree (Fig. 1a).

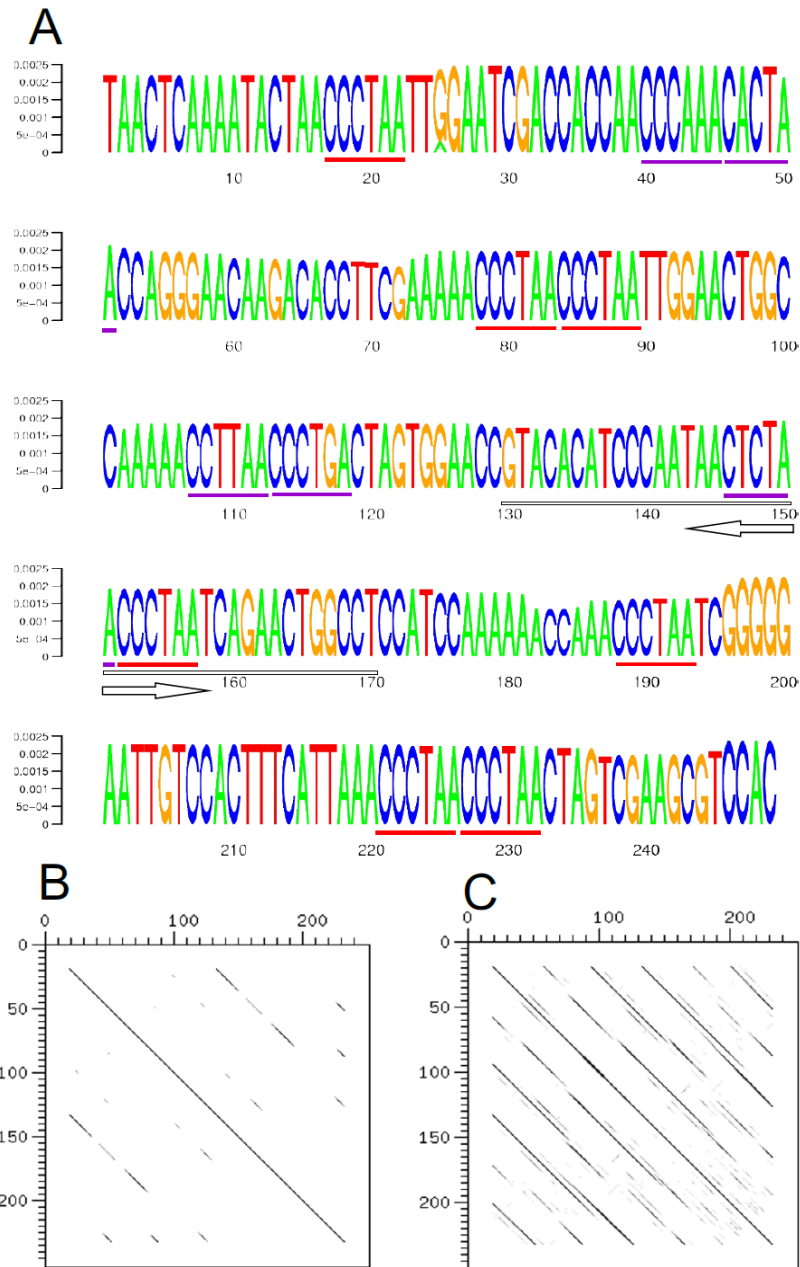


Fig. 1 *PaB6* monomer characterization. **a** Monomer sequence logo (Schneider and Stephens 1990) with height of the letters corresponding to *k*-mer frequencies. Arrows indicate beginning and direction of forward and reverse primers (underlined). Perfect telomeric sequences are underlined in red, and imperfect variants in violet. **b–c** dot plots of the monomer sequence against itself with lower (**b**) and higher similarity stringency (**c**).

Comparative sequence analysis of the monomers

PCR amplification of major type of the monomer using primers designed for reconstructed B^6 -genome monomer resulted in products of expected length in all the 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 approximately 250 bp, corresponding to the *PaB6* monomer (Fig. 2a), a second band of c. 120–130 bp and, occasionally, a third band of c. 60–80 bp. Main bands of c. 249 bp, corresponding to the expected size of the monomer of *PaB6*, were isolated, cloned, and sequenced from two or three individuals of each of the six cytotypes. Amplification of dimers or even longer fragments was not observed or observed rarely.

The outgroup taxa, from outside of the genus *Prospero*, but of family Hyacinthaceae, showed no amplification with the same PCR amplification protocol and primers. Representatives of more distantly related genera, *Dipcadi* sp. and *Othocallis siberica*, showed no bands after PCR amplification, regardless of the annealing temperature. The more closely related *Barnardia scilloides* and *Schnarfia messeniaca* produced very faint, monomer-related bands, on the border of detection, and only when a lower annealing temperature was used (Fig. 2b and not shown).

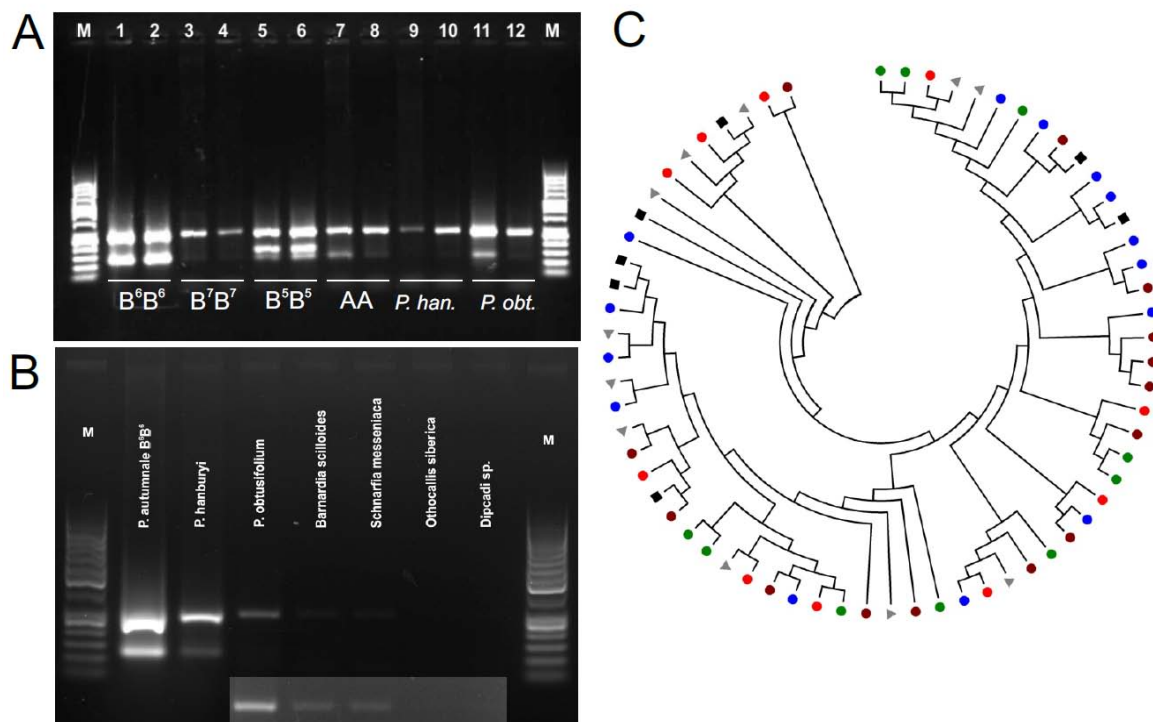


Fig. 2 Patterns of PCR amplification of *PaB6* satellite DNA in *Prospero* and comparative phylogenetic analysis of major monomer sequence. **a** PCR amplification products of *PaB6* monomers (M– marker; 1–2: B⁶B⁶ (H166, H427); 3–4: B⁷B⁷ (H424, H428); 5–6: B⁵B⁵ (H582, H640); 7–8: AA (H541, H550); 9–10: *P. hanburyi* (H397, H115); 11–12: *P. obtusifolium* (H559, H563; Supplementary Table S1). **b** PCR amplification of *PaB6* monomers in selected *Prospero* samples and outgroup taxa (annealing temperature 55°C); inset shows contrasted image with weak bands of amplification in *Barnardia* and *Schnarfia*. **c** Neighbor joining tree of *PaB6* repeats cloned from diploid cytotypes of *P. autumnale* (circles; AA, green; B⁵B⁵, red; B⁶B⁶, blue; B⁷B⁷, brown), *P. obtusifolium* (grey triangles), and *P. hanburyi* (black squares).

Sequence analysis of cloned *PaB6* monomers (66 sequences; Table S1), representing monomers amplified from two or three individuals of each of the six diploid taxa, confirmed that they all carried reconstructed *PaB6* repeats. Fifty-one amplified monomers (83%) were 249 bp long, with twelve shorter (18%; 119 bp, 175 bp, 243 bp, 247 bp and 248 bp; due to deletions) and three longer (4.5%; 250 bp and 256 bp, latter due to a TTAGGG insertion). High overall levels of sequence similarity amongst the amplified population *PaB6* monomers both within (93–100%) and between (92–100%) the different diploid cytotypes of *P. autumnale* and two other *Prospero* species, were observed (Table S2). Thus, the inter-cytotype sequence variation of repeats amplified with specific reconstructed monomer primers was as equally low and random as that within cytotypes or between individuals. It was mostly due to single base-pair indels or point mutations occurring at different positions along the monomer, and being monomer-specific (alignment available upon request).

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

Genomic organization and copy number variation of *PaB6*

Southern blot hybridization, using the satellite DNA single repeat (monomer) isolated from cytotype B⁶B⁶ as probe, allowed analysis of genomic organization and estimates of copy number of *PaB6* repeats to be made. The probe hybridized strongly to genomic DNA of cytotype B⁶B⁶ (Fig. 3a–c), moderately to cytotype B⁵B⁵ (Fig. 3b, c), and weakly to some individuals of cytotype B⁷B⁷ (Fig. 3a, b, d). No or very weak signal was detected genomic DNA of cytotype AA (Fig. 3b), *P. hanburyi* or *P. obtusifolium* (Fig. 3b) after hybridization with the *PaB6* probe.

The hybridization pattern of *PaB6* was typical of tandemly repeated DNA, with the major 249 bp band and its multiples being most prominent in all samples. Additional, weaker band about 375bp in length, corresponding to an additional major subunit identified in *PaB6* (Fig. 3b, c) has also been detected in all samples. The length of this additional band was slightly shifted in two B⁵B⁵ individuals and in an AB⁵ diploid hybrid (not shown).

Methylation of *PaB6* repeats was analyzed in cytotype B⁶B⁶, after digestion with methylation-insensitive (*Bst*NI) and methylation-sensitive (*Scr*FI) restriction enzymes with the same recognition site. The satDNA monomers were heavily methylated (Fig. S3).

Copy numbers of *PaB6* were estimated by quantitative chemiluminescent dot blot hybridization of labelled *PaB6* as probe against known quantities of genomic DNAs of all six cytotypes. Large differences in the genomic content of *PaB6* between the four cytotypes of *P. autumnale* corroborated the results of Southern blot experiments and FISH (see below). The highest copy number was found in cytotype B⁶B⁶ (1763–2058 copies per haploid genome; 7–10%), followed by cytotype B⁵B⁵ (1153–1373 copies/1C; 6–7%), one accession of cytotype B⁷B⁷ (21–25 copies/1C; c. 0.13%), and AA (20–27 copies/1C; c. 0.08%; Table 1; Fig. 3b). *P. obtusifolium* and *P. autumnale* possessed very only very low amount of *PaB6* which could not be determined.

Chromosomal localization and organization of *PaB6* repeats

PaB6 has been localized in all six cytotypes using FISH (Table 2). The variation in number and size of satDNA loci detected coincided with the Southern slot results. *P. obtusifolium* (Fig. 4a) and *P. hanburyi* (Fig. 4b) had no detectable *PaB6* loci. PCR recovered copies of *PaB6* from these two genomes, but Southern blotting (Fig. 3b) indicated very low copy numbers, presumably below the detection limit both Southern blotting and FISH. How these sequences are organized is unclear.

Table 1 Characterization of satellite *PaB6* repeats in diploid cytotypes and species of genus *Prospero*.

Taxon	2n	PCR amplification of <i>PaB6</i>	% of the genome	Copy number/1C	Genome size (pg per 1C; Jang et al., 2013)
<i>P. autumnale</i>					
cytotype B ⁵ B ⁵	10	+	6.3-7.4%	1153-1373	4.86 ± 0.002
cytotype B ⁶ B ⁶	12	+	7.16-10.71%	1763-2058	6.27 ± 0.083
cytotype B ⁷ B ⁷ : single 5S ¹ rDNA	14	+	—**	—**	4.23 ± 0.048
cytotype B ⁷ B ⁷ : duplicated 5S ¹ rDNA	14	+	0.12-0.14%	21-25	4.45 ± 0.023
cytotype AA	14	+	0.07-0.09%	20-27	7.85 ± 0.045
<i>P. hanburyi</i>	14	+	n.d.*	n.d.*	6.81 ± 0.017
<i>P. obtusifolium</i>	8	+	n.d.*	n.d.*	4.94 ± 0.039

*copy number could not be determined due to very low *PaB6* contents; **not determined due to lack of plant material (*Prospero* enters rest phase from April till October and does not produce any leaves during this time).

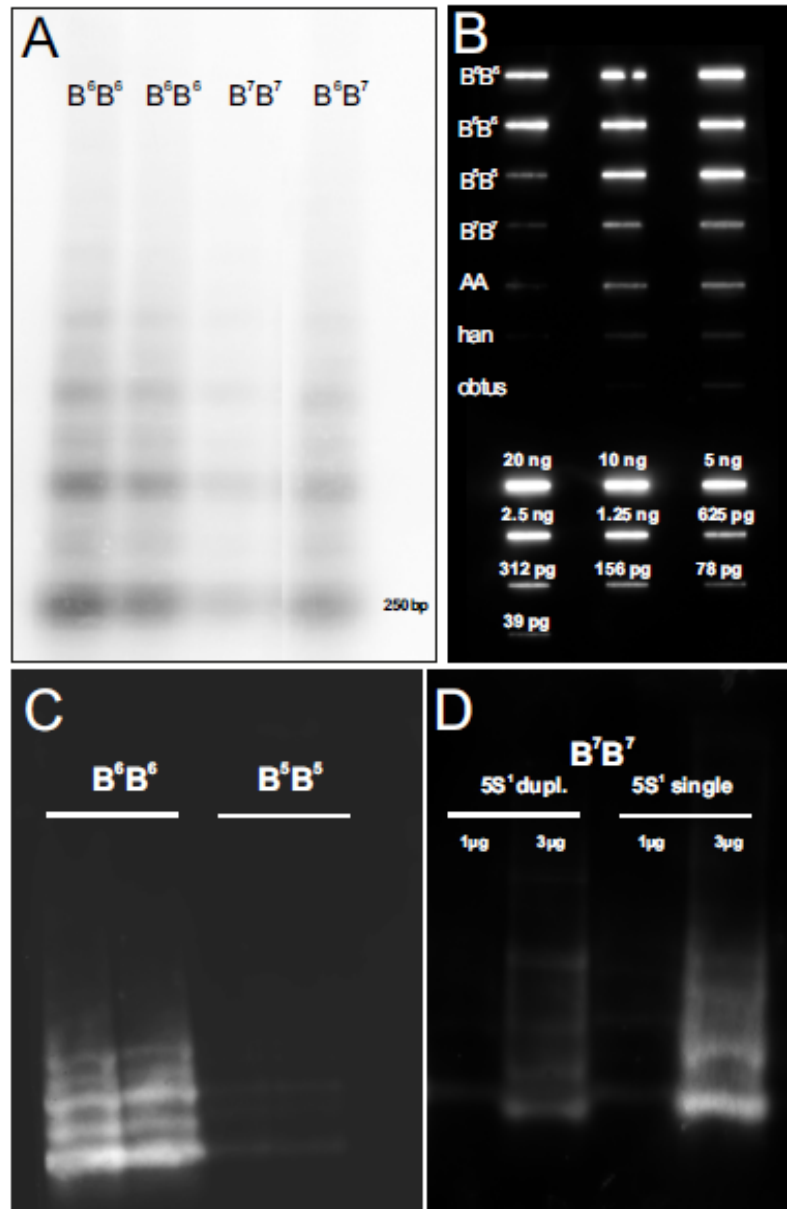


Fig. 3 Analyses of genomic organization of *PaB6* repeats in *Prospero* using Southern blot (**a**, **c**, **d**) hybridization and slot blotting used for copy number estimation (**b**). **a** radioactive *in situ* hybridization of *PaB6* to genomic DNA of cytotype B^6B^6 (H166; H271), B^7B^7 (H428), and diploid homoploid hybrid B^6B^7 (H294). **b** slot blot for *PaB6* copy number determination (Supplementary Table S1). **c–d** chemiluminescent detection of dig-labelled *PaB6* probe in restricted genomic DNA. **c** B^6B^6 (H166, H468), B^5B^5 (H637, H565). **d** with 1 μ g and 3 μ g DNA, B^7B^7 : H424 (duplicated 5S¹ rDNA); B^7B^7 : H428 (single 5S¹ rDNA).

P. autumnale diploids, by contrast, all exhibited hybridization signals, but of variable numbers and 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 (Fig. S2). In cytotype B^6B^6 , major *PaB6* loci were present in all chromosomes of the complement (Fig. 4f, g). The pattern of satellite distribution in individuals and

populations was remarkably uniform, and all satellite loci were of similar signal strength. The only polymorphism was in chromosome one, where one locus was heterozygous and occasionally varied in size between homologues (Fig. 4f, g). *PaB6* loci occurred on four of the five chromosome pairs of cytotype B⁵B⁵ (Fig. 4h) and were of similar signal strength. Chromosome 3 showed, at most, a very weak hybridization signal (not shown; Table 2).

Table 2 Characterization of 5S rDNA and satellite DNA *PaB6* loci in chromosomes of diploid species and cytotypes of genus *Prospero*.

Cytotype	Number and locations of 5S rDNA loci	Number and locations of <i>PaB6</i> loci	Signal strength	Figure
<i>Prospero obtusifolium</i> (2n = 8)	3: L-P ² , L-P ³ , S-P ³	–	–	4A, 5A
<i>P. hanburyi</i> (2n = 14)	1: L-P ¹	–	–	4B, 5B
<i>P. autumnale</i> complex AA (2n = 14)	1: S-P ²	1: S-P ⁵	Very weak	4C, 5C
B ⁷ B ⁷ (2n = 14)	1: L-D ^{1**}	3: S-P ^{1, 2, 4}	+	4D, 5D
	1: L-D ^{1*}	6: S-P ^{1, 2, 4, 5, 6, 7}	++	4E, 5E
B ⁶ B ⁶ (2n = 12)	2: L-D ¹ , S-P ²	6: S-P ^{1-hetero, 2, 3, 4, 5, 6}	+++++	4F
	2: L-D ¹ , S-P ²	6: S-P ^{1, 2, 3, 4, 5, 6}	+++++	4G, 5F
B ⁵ B ⁵ (2n = 10)	1: L-D ¹	4: S-P ^{1, 2, 4, 5}	+++	4H
	1: L-D ¹	5: S-P ^{1, 2, 3-hetero, 4, 5}	+++	
Diploid homoploid hybrids AB ⁵ (2n = 12)	1 (A): S-P ² ; 1 (B ⁵): L-D ¹	4 (B ⁵): S-P ^{1, 2, 4, 5}	–;++++	4I, 5G
B ⁵ B ⁷ (2n = 12)	1 (B ⁵): L-D ¹ ; 1 (B ⁷): L-D ^{1*}	4 (B ⁵): S-P ^{1, 2, 4, 5} ; 6 (B ⁷): S-P ^{1, 2, 3, 4, 5, 6}	++++;+	4J
B ⁶ B ⁷ (2n = 13)	2 (B ⁶): L-D ¹ , S-P ² ; 1 (B ⁷): L-D ^{1*}	6 (B ⁶): S-P ^{1, 2, 3, 4, 5, 6}	+++++; –	4K, 5H

Note: S, short arm; L, long arm; P, pericentric region; D, distal region; ¹⁻⁷, number of the chromosome; **, duplicated 5S¹ rDNA signal; *, single 5S¹ rDNA signal; +, ++, +++ etc., strength of *PaB6* signals; – no *PaB6* signals. hetero, heterozygous locus.

The most variable *PaB6* distribution was shown by cytotype B⁷B⁷. Some individuals possessed moderately amplified loci in pericentromeric regions of all chromosomes (Fig. 4e; Table 2), while others had weakly amplified loci of *PaB6* in only three chromosome pairs (Fig. 4d; Table 2). These patterns correlated with a duplication polymorphism of 5S rDNA present on chromosome 1 (5S¹; Fig. 6). Thus five individuals with a single 5S¹ rDNA locus showed strongly amplified satDNA loci on all chromosomes (Fig. 4e), while six plants with a duplicated 5S locus carried *PaB6* loci only on chromosomes 1, 2 and 4 (Fig. 4d). Cytotype AA had a single locus on chromosome 5, but this was weak and barely detectable (Fig. 4c). All six diploid homoploid hybrids (all F₁ generation) possessed perfectly additive numbers and strengths of satellite DNA loci compared to the diploid parents. The Southern blot hybridization pattern of *PaB6* in the genome of one of the hybrids of B⁶B⁷ origin also indicated its additivity (Fig. 3a). The number and localization of *PaB6* satDNA loci in all cytotypes is summarized in Table 2.

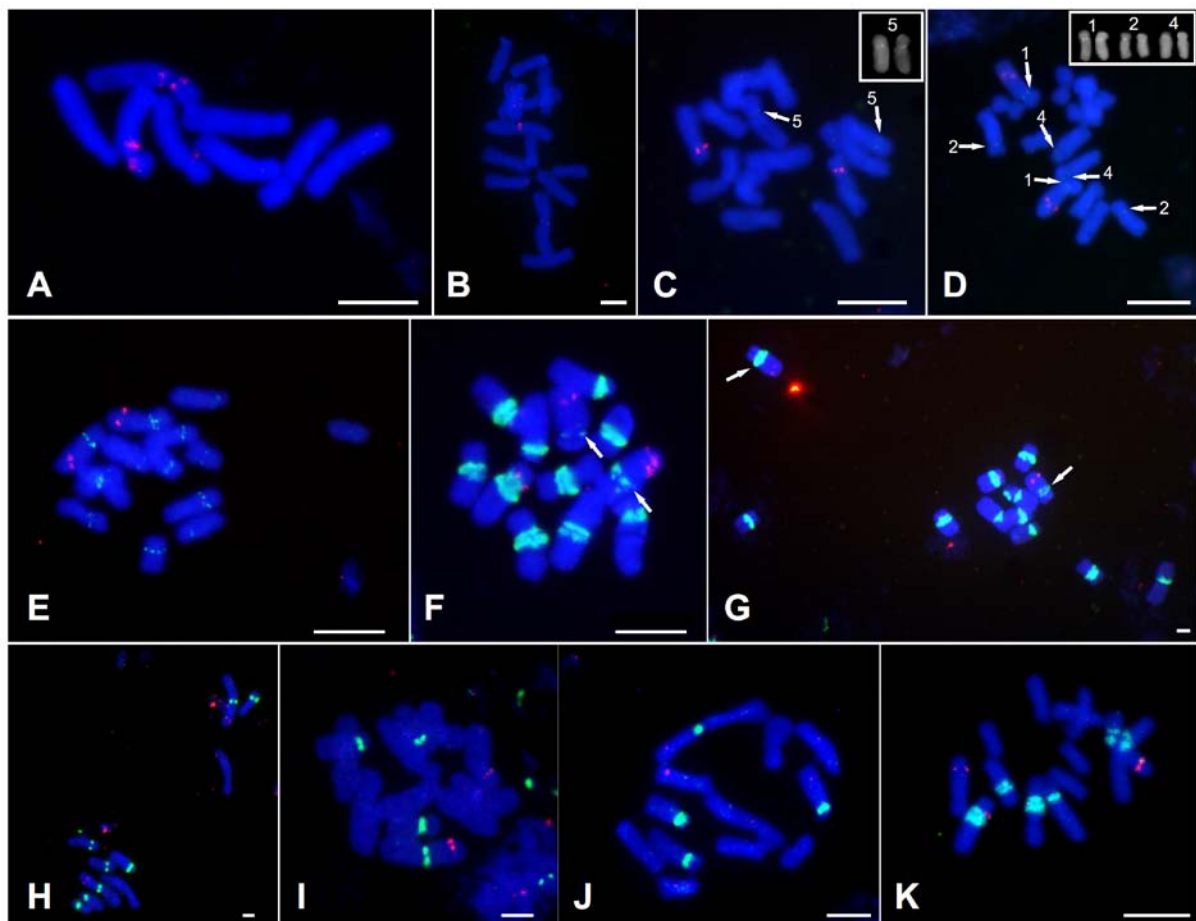


Fig. 4 Localization of *PaB6* in chromosomes of diploid *Prosopis* species and cytotypes, and three homoploid hybrids. **a** *P. obtusifolium*. **b** *P. hanburyi*. **c–k** *P. autumnale* complex. **c** cytotype AA. **d–e** B^7B^7 with duplicated (**d**) and single (**e**) 5S rDNA locus in chromosome 1. **f–g** B^6B^6 with weak (**f**) and strong (**g**) signal of *PaB6* in chromosome 2 (arrows). **h** B^5B^5 . **i** AB^5 . **j** B^5B^7 . **k** B^6B^7 . Insets: chromosomes carrying *PaB6* signals.

The *PaB6* monomer contains seven perfect and a few imperfect vertebrate-type telomeric sequences (TTAGGG), typical of the monocot order Asparagales. TTAGGG sequences were detected at chromosome ends (Fig. 5) but were additionally also co-localizing with satellite DNA *PaB6* sites. The signal intensity of telomeric PNA probe in pericentric chromosome regions corresponds nearly perfectly to signal strength and localization of *PaB6* probe (Figs. 4 and 5).

Discussion

Higher order structure and chromosomal localization of a newly isolated satellite DNA

Analysis of satellite DNA *PaB6*, isolated from the diploid B⁶B⁶ cytotype of *Prospero autumnale*, has allowed us to make inferences about the evolution of the two species and the species complex which comprise the genus. Following its detection through NGS, the repeat has been analyzed to characterize its monomers, their chromosomal locations, methylation status, and extent of amplification through copy number estimations, in all diploid species and cytotypes of the genus. This has also enabled the origin and evolution of the satDNA itself to be determined.

PaB6 has a 249 bp monomer, which represents a higher-order structure composed of two major imperfect subrepeats, each of which consists of three secondary minor subrepeats. Southern blot *in situ* hybridization and PCR amplification emphasized this complexity, both methods revealing two weaker subunits. This suggests that the specific primer binding sites have been retained in those repeats, although somewhat depauperate. Satellite *PaB6* is highly methylated in cytotype B⁶B⁶, and co-locates with the pericentric heterochromatic blocks detected in this cytotype by Ebert et al. (1996). Satellite DNAs with such higher order structures have been isolated and characterized from a few other plant genomes (Ambrožová et al. 2011; Iwata et al. 2013), although the majority reported are simple monomers (Hemleben et al. 2007; Plohl et al. 2010). Complex higher order structures, such as this one, evolve from simple monomers, some of which may remain in the genome and then amplify independently (Iwata et al. 2013).

Satellite DNA *PaB6* constitutes about 8–10% of genome B⁶, and is located in pericentric regions of all chromosomes. Typically, plant satDNA is localized in pericentric regions or is subtelomeric, sometimes being chromosome specific (Hemleben et al. 2007; Plohl 2010), although occasional interstitial sites have been found (Hemleben et al. 2007). There is no evidence that *PaB6* plays a role in centromere function. However, no centromere-specific sequences have yet been identified among potential candidates (retrotransposons, other satellite DNAs) generated via NGS of the *Prospero* repetitive genome fraction (Weiss-Schneeweiss, Macas et al. unpublished data). *PaB6*, then, may be one of the components of the centromere whether or not playing a role in its function. Studies of centromere structure in *Prospero* will address this issue further.

The *PaB6* sequence showed no similarity to genomic regions (rDNA, transposable elements) available in public databases (SatDNA, GenBank). It did, however, contain two perfect dimers and several imperfect repeats of the vertebrate-type telomeric sequence TTAGGG, which typifies the telomeres of the monocot order Asparagales to which *Prospero* belongs (Sýkorová et al. 2003; Weiss-Schneeweiss et al. 2004). The presence of interstitial

telomeric repeats (ITRs) is often interpreted as a remnant of evolution by telomere-telomere chromosomal fusions, but 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 arms inversions involving centromere and telomere (Presting et al. 1996). The occurrence of telomeric repeats within, or at the margins of, constitutive heterochromatin has mainly been reported from vertebrates (Meyne et al. 1990) but is also known in plants (Presting et al. 1996; Uchida et al. 2002; Weiss-Schneeweiss et al. 2004; Gong et al. 2012; He et al. 2013). It has been argued that telomeric repeats can be an integral and long-established part of satDNAs of constitutive heterochromatin (Slijepcevic et al. 1996; Garrido-Ramos et al. 1998; Metcalf et al. 2004), originally inserted and amplified in interstitial chromosomal positions through DNA double strand breaks (DSBs) repair by telomerase (Nergadze et al. 2004, 2007).

ITR have been reported in pericentric or centromeric regions in several higher plant genera (Presting et al. 1996; Uchida et al. 2002; Gong et al. 2012; He et al. 2013). Pericentric localization of *PaB6* in *Prospero* chromosomes and its enrichment in ITR particularly mirrors general patterns reported in genus *Solanum* (Gong et al. 2012; He et al. 2013). Megabasepair-sized ITRs in *Solanum* were shown to be associated with a satellite DNA identified in functional domains of potato chromosomes (He et al. 2013). In contrast, however, ITRs detected in *Prospero* are integral part of *PaB6* tandem repeats and are interspersed with other sequence motifs. Origin of these cannot be unambiguously established.

The number and distribution of telomeric repeats in monomers of *PaB6* is clearly sufficient to allow their localization using a PNA telomeric probe (CCCTAA)₃. The FISH patterns reflected those detected with the *PaB6* probe itself. By contrast, the longer but less sensitive DNA telomeric probe (TTAGGG)₃₃₋₅₀ (Weiss and Scherthan 2002; Weiss-Schneeweiss et al. 2004) hybridized only to the telomeres of *Prospero* (data not shown). Thus, telomeric repeats may be more common in plant genomes than currently assumed but remain undetected when present in lower copy number or interspersed with other sequence motifs as a part of satDNA monomers.

Hypothesis on the origin of *PaB6*

Very weak amplification of *PaB6* monomer-equivalent bands has been found in *Barnardia* and *Othocallis*, genera closely related to *Prospero* (Pfosser and Speta 1999; Ali et al. 2012). This suggests an early evolutionary origin of this higher-order repeat monomer and its

maintenance in the genomes of *Prospero* ancestors, accompanied by the accumulation of mutations and homogenization of the repeat. Ancestors of *Barnardia* and *Prospero* diverged about 15.3 mya (Ali et al. 2012), which fixes a minimum age for *PaB6*. Screening of other related genera would allow a more precise estimate of monomer sequence composition. Amplification of *PaB6* in the outgroup taxa was too weak to allow further analyses to be made. The telomeric repeats present in the *PaB6* monomer may have originated as a remnant of chromosomal rearrangements predating the emergence of the genus *Prospero*. Such remnant telomeres, located near centromeres in a new genomic environment, may have co-evolved with their flanking sequences to give rise to a small population of repeats. Thus *PaB6* likely evolved before the emergence of the genus *Prospero*.

Evolutionary dynamics of *PaB6* in *Prospero*: parallel amplification coincides with independent fusion events

PaB6 likely evolved before the emergence of the genus *Prospero*, and remained in low copy number as part of the library of repeats (Meštrovič et al. 1998) in genomically stable *P. obtusifolium* and *P. hanburyi*. Amplification of *PaB6* began only in chromosomally dynamic *P. autumnale*, possibly stimulated by the high incidence of chromosomal rearrangements (see below). The dynamics of different families of satDNAs can vary both among related species and among families of satellite present in the same genome, with some satDNAs diversifying within a genome accompanying certain events of speciation and (Quesada del Bosque et al. 2011), while others remain unchanged throughout long evolutionary periods (Vershinin et al. 1996; Ugarković and Plohl 2002).

The evolutionary dynamics of the *PaB6* repeat can be assessed against a clear phylogeny of the genus (Jang et al. 2013). *PaB6* has been amplified in all three species of *Prospero*, albeit to different extents. Thus *P. obtusifolium* and *P. hanburyi* possess very few copies and these can only be detected by PCR, since they are below the detection limit of FISH and Southern blotting, and on the border of detection of slot blots. The four diploid cytotypes of the *P. autumnale* complex, by contrast, all possess *PaB6* in unique amounts detectable by FISH, ranging from 20 copies per 1C genome in AA to 2058 copies in B⁶B⁶. The correlation between intensity of FISH signal and copy number is very strong. Copy number variation has been observed only between the two chromosomally differentiated lineages of the widespread cytotype B⁷B⁷ - with either a single or a duplicated 5S rDNA locus in chromosome 1 (5S¹) - characterized so far. Plants with a single 5S¹ rDNA possess higher *PaB6* copy numbers in all

chromosomes than plants with a duplicated 5S¹ locus, in which only three chromosome pairs show *PaB6* sites. The geographically restricted cytotypes B⁵B⁵ (Libya), B⁶B⁶ (Crete), and AA (Spain, Portugal and Morocco) are phylogenetically more cohesive and also show no or little variation in *PaB6* copy numbers, such as heterozygosity of locus on chromosome 1 in cytotype B⁶B⁶.

PaB6 is very strongly amplified in cytotypes B⁶B⁶ (8–10% of the genome) and B⁵B⁵ (6–7% of the genome). These chromosome numbers evolved via independent fusion events from different lineages of the B⁷B⁷ cytotype, and so are not directly related to one another phylogenetically (i.e. not in a sister relationship; Jang et al. 2013; Fig. 6). Both, however, show strong amplification of *PaB6* above the B⁷ genome level. This parallel amplification of *PaB6* might have been triggered by, or associated with, the chromosomal fusions and their accompanying chromosomal rearrangements (such as inversions; Ainsworth et al. 1983; Vaughan et al. 1997; Jang et al. 2013). Chromosome rearrangement-triggered amplification of *PaB6* satellite is particularly plausible for the phylogenetically young cytotype B⁵B⁵, which is embedded within B⁷B⁷, a cytotype carrying relatively few copies of *PaB6* (Jang et al. 2013, Fig. 6). Cytotype B⁶B⁶, while clearly originating from $x = 7$, does not strongly tie, phylogenetically or chromosomally, to any lineage of present-day B⁷B⁷ and may have arisen from the ancestral cytotype inferred to resemble present day genome B⁷ (Jang et al. 2013). *PaB6* was likely present in only low copy number in the ancestral karyotype, suggesting rearrangement-associated or -driven amplification of *PaB6* in the evolution of B⁶B⁶. Alternatively, B⁶B⁶ may have originated from an extinct, or even extant, lineage of B⁷B⁷ possessing significant copy numbers of the *PaB6* repeat. Population sampling of B⁷B⁷, especially in the eastern Mediterranean region, is under way to explore this possibility. Similar convergent amplification of satellite DNAs in different lineages of closely related taxa, at different evolutionary times has been reported in other taxa (Navajas-Pérez et al. 2009; Quesada del Bosque et al. 2011; Rosato et al. 2012).

The present data do not allow mechanisms leading to *PaB6* amplification to be inferred. However, temporary instability of the centromeric region of fusion chromosomes may have promoted *PaB6* amplification in cytotypes B⁶B⁶ and B⁵B⁵, followed by spread throughout the rest of the complement. Two mechanisms for satDNA copy number change are generally acknowledged unequal crossing-over with gene conversion (Liao 1999; Eickbush and Eickbush 2007), or amplification and homogenization of monomers by extrachromosomal circular DNA (eccDNA, “rolling circle”) molecules during recombination (Navrátilová et al. 2008; Cohen et al. 2010). Satellite repeat-derived eccDNAs are ubiquitous in plants (Cohen

et al. 2008; Navrátilová et al. 2008; Cohen and Segal 2009). These two mechanisms are not mutually exclusive, and might operate in concert resulting in mobility and homogenization of repetitive DNAs. Whether these mechanisms are also involved in expansion of *PaB6* in *Prospero* remains unknown. Diploid homoploid hybrids might offer insight into the mechanisms of *PaB6* mobility in the genome. All three genomic hybrid combinations (representing F₁ generation) possess strictly additive parental patterns of *PaB6* copy number and distribution in chromosomes. At least some of the hybrids were shown to undergo homoeologous bivalent chromosome pairing in meiosis. Analyses of meiotic process and meiotic products might shed light onto the mechanisms and rates of *PaB6* amplification.

Although copy number varies significantly among cytotypes within *Prospero*, the repeats identical or very similar to the reconstructed *PaB6* monomer are found across the genus. Such strong sequence conservation may indicate recent amplification of this variant of the monomer, so that mutations did not accumulate and got fixed yet or it may indicate efficient systems of sequence homogenization and gene flow between taxa (Hemleben et al. 2007). Our data, coupled with the geographically disjunct distribution of cytotypes AA, B⁵B⁵, and B⁶B⁶, and lack of gene flow among these suggest rather recent multiple and independent rounds of amplification of *PaB6* from a small pool of ancestral repeats (Mravinac et al. 2005; Plohl et al. 2010). The presence of other variants of monomers of *PaB6* in some or all cytotypes cannot be excluded and screening of those has been initiated.

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Supplementary Table S1 Plants used in this study with collection details, GenBank accession numbers (*PaB6*), and methods used for analysis. All plants cultivated in HBV (Vienna, Austria).

Taxon (chromosome number, cytotype)	Locality; Collection; accession number	Methods applied	GenBank accession number ⁴
<i>P. hanburyi</i> ($2n = 14$)	Turkey, Findikpinar; Speta; H115	PCR, Seq, SB, FISH	
	Turkey, Findikpinar; Speta; H397	PCR, Seq, SB, FISH ¹	
	Turkey, Narlikuyu, Silifke; H231	FISH, CNE	
<i>P. obtusifolium</i> ($2n = 8$)	Morocco; Parker; H547	Seq, SB	
	Morocco; Parker; H563	PCR, FISH	
	Morocco; Parker; H564	FISH	
	Spain; Parker; H559	PCR, Seq, SB	
	Spain; Parker; H540	SB, FISH ¹ , CNE	
<i>P. autumnale</i>			
($2n = 14$, AA)	Spain, Huelva; Parker; H541	PCR, Seq, SB	
	Spain, Badajoz; Parker; H543	Seq, SB, FISH ¹	
	Spain, Jaen; Parker; H546	SB	
	Spain, Huelva; Parker; H548	SB, FISH, CNE	
	Portugal, Peniche; Parker; H550	PCR, SB, FISH	
	Portugal, Peniche; Parker; H551	FISH	
	Portugal, Peniche; Parker; H552	SB	
($2n = 10$, B ⁵ B ⁵)	Libya, Mt. Tob; Parker; H566	Seq, SB, CNE	
	Libya, Mt. Tob; Parker; H581	FISH	
	Libya, Mt. Tob; Parker; H582	PCR, Seq, SB	
	Libya, Nagasa; Parker; H640	PCR, FISH	
($2n = 12$, B ⁶ B ⁶)	Greece, Crete; Speta; H166	PCR, Seq	
	Greece, Crete; Speta; H170	SB, FISH	
	Greece, Crete; Speta; H195	Seq, SB, FISH, CNE	
	Greece, Crete; Speta; H271	SB	
	Greece, Crete; Speta; H274	SB, FISH	
	Greece, Crete; Speta; H427	PCR, Seq, SB, FISH, CNE	
	Greece, Crete; Speta; H455	SB	
	Greece, Crete; Speta; H468	FISH ¹	
($2n = 14$, B ⁷ B ⁷)	Greece, Crete; Speta; H47	SB, FISH ³	
	Greece, Crete; Speta; H58	Seq, SB	
	Greece, Crete; Speta; H89	Seq, SB	
	Greece, Crete; Speta; H174	SB	
	Greece, Crete; Speta; H440	SB, FISH ^{1, 3}	
	Greece, Crete; Speta; H464	SB	
	Greece, Lefkas, Nikiana; Speta; H432	SB	
	Greece, Kalamitsi; Speta; H450	SB, FISH ³	
	Greece, Poros; Speta; H535	Seq, SB	
	Greece, Rhodes; Speta; H137	FISH ²	
	Israel, Nene Han; Parker; H612	FISH ³	
	Italy, Sicily; Speta; H428	PCR, SB, FISH ³	
	Italy, Sicily; Speta; H403	SB	
	Montenegro; Speta; H422	FISH ^{1, 2}	
	Montenegro; Speta; H424	PCR, SB, FISH ^{1, 2} , CNE	
	Montenegro; Speta; H430	SB	
	Serbia, Siget-Baum; Rat; H576	FISH ²	
	Turkey; Speta; H179	SB	
	Ukraine, Nikita; Kish; H591	FISH ²	
	Speta; H228	FISH ²	
	Speta; H433	SB	
Diploid homoploid hybrids			
($2n = 12$, AB ⁵)	Cross pollination experiment; Parker; H567	SB, FISH ¹	
($2n = 12$, B ⁵ B ⁷)	Cross pollination experiment; Parker; H633	FISH	
($2n = 13$, B ⁶ B ⁷)	Greece, Mykonos; Speta; H294	SB	
	Greece, Crete; Speta; H211	FISH ¹	
	Greece, Crete; Speta; H518	FISH	
	Greece, Crete; Speta; H700	FISH	
Outgroups			
<i>Barnardia scilloides</i>	Cult. HBV; Bj3-6	PCR	
<i>Schnarfia messeniaca</i>	Cult. HBV; Hyac639	PCR	
<i>Dipca di</i> sp.	Cult. HBV; Hyac336	PCR	
<i>Othocallis siberica</i>	Cult. HBV; Hyac2159/1	PCR	

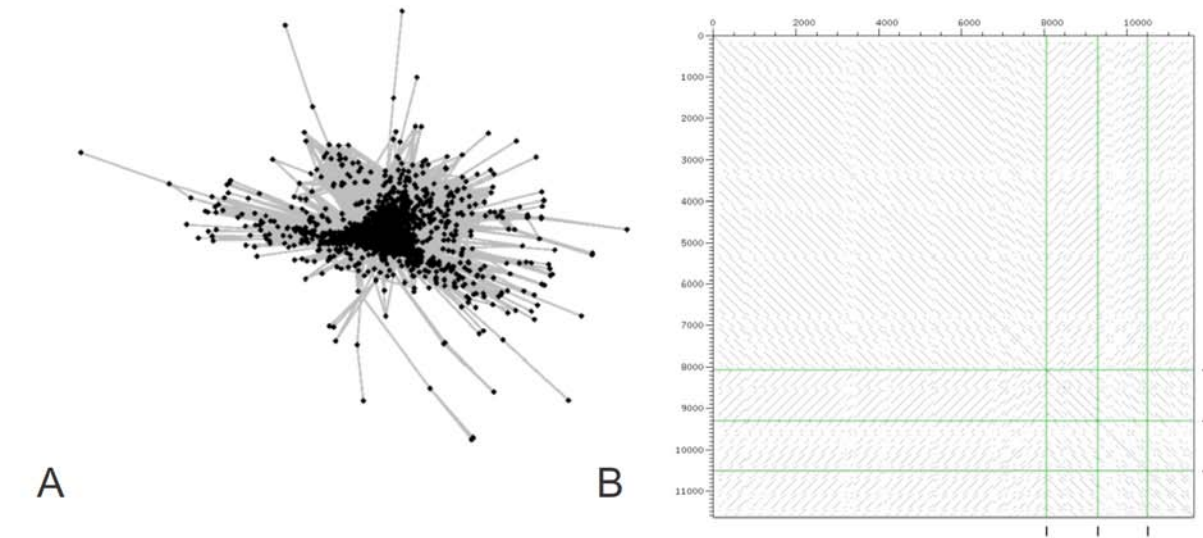
Seq, Sequencing; SB, Southern blot; DB, Dot blot; FISH, Fluorescence *in situ* hybridization with 5S rDNA and *PaB6*; CNE, copy number estimation via slot blot; ¹, FISH with PNA telomeric probe (CCCTAA)₃; ² duplicated and ³ single locus of 5S rDNA in chromosome 1; ⁴ in preparation.

Supplementary Table S2 Sequence similarity (%) of cloned monomers of *PaB6* satellite DNA within and between different diploid cytotypes of *Prospero* given as: (minimum), average $\pm$ SD, (maximum).¹

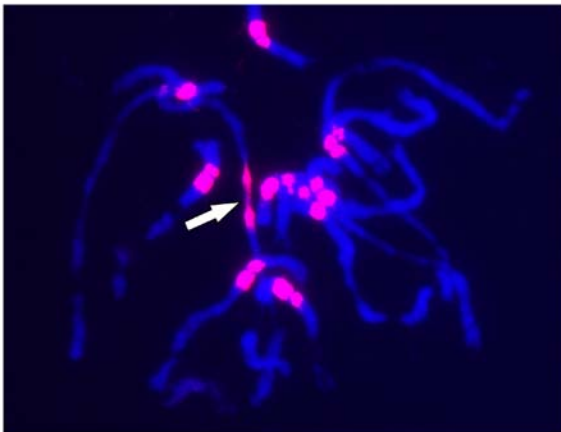
	<i>P. autumnale</i> B ⁷ B ⁷	<i>P. autumnale</i> B ⁶ B ⁶	<i>P. autumnale</i> B ⁵ B ⁵	<i>P. autumnale</i> AA	<i>P. hanburyi</i>	<i>P. obtusifolium</i>
B ⁷ B ⁷	(95) 98.4 $\pm$ 1.2 (100)					
B ⁶ B ⁶	(94) 98.0 $\pm$ 1.3 (100)	(95) 96.5 $\pm$ 0.9 (99)				
B ⁵ B ⁵	(92) 97.5 $\pm$ 1.6 (100)	(94) 96.6 $\pm$ 1.1 (99)	(94) 96.5 $\pm$ 1.2 (99)			
AA	(95) 98.3 $\pm$ 1.1 (100)	(94) 96.8 $\pm$ 1.2 (100)	(94) 96.5 $\pm$ 1.3 (100)	(94) 96.4 $\pm$ 1.5 (100)		
<i>P. hanburyi</i>	(94) 97.8 $\pm$ 1.5 (100)	(94) 96.2 $\pm$ 1.0 (100)	(94) 96.1 $\pm$ 1.2 (100)	(94) 96.5 $\pm$ 1.3 (100)	(95) 96.0 $\pm$ 1.3 (100)	
<i>P. obtusifolium</i>	(95) 98.1 $\pm$ 1.2 (100)	(93) 96.4 $\pm$ 1.2 (100)	(93) 96.5 $\pm$ 1.4 (100)	(93) 96.6 $\pm$ 1.6 (100)	(93) 96.2 $\pm$ 1.3 (100)	(93) 96.4 $\pm$ 1.5 (100)

Comparison of all vs. all: (92) 97.55 $\pm$ 1.5 (100).

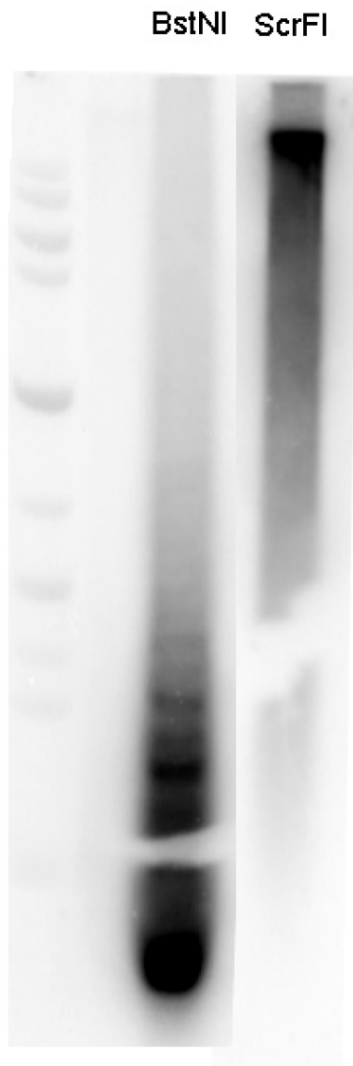
Supplementary Fig. 1 **a** Graph layout (cluster CL0009) identified by NGS. **b** dot plot of the sequences of longest contigs of cluster 9 indicating the presence of tandem repeats in head-to-tail orientation.



Supplementary Fig. 2 Distribution of *PaB6* in pericentric and centromeric regions on all chromosomes in cytotype B⁶B⁶. Arrow indicates *PaB6* signal in extended centromeric region.



Supplementary Fig. 3 *PaB6* in the B⁶ genome (H247) restricted with *Bst*NI and *Scr*F1, showing DNA high methylation of monomers.



–CHAPTER 3–

More than meets the eye: numerical convergence, cycles of hybridization, and contrasting evolutionary trajectories in polyploids of the *Prospero autumnale* complex (Hyacinthaceae)

Tae-Soo Jang¹, Khatere Emadzade¹, Eva M. Temsch¹, John Parker², Jiří Macas³, Andrew R. Leitch⁴, Franz Speta⁵, Hanna Weiss-Schneeweiss¹

¹Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria;

²Cambridge University Botanic Garden, Cambridge, CB2 1JF, UK;

³Institute of Plant Molecular Biology, CAS, Ceske Budejovice, Czech Republic;

⁴Queen Mary College, University of London, London, UK;

⁵Dornacher Strasse 1, 4040 Linz, Austria

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Abstract

The Mediterranean *Prospero autumnale* complex (Hyacinthaceae) comprises four evolutionary distinct diploid lineages – AA ($x = 7$), B^7B^7 ($x = 7$, with two distinct lineages), B^6B^6 ($x = 6$), and B^5B^5 ($x = 5$) – each having a unique combination of chromosomal and genomic features. Three of the four diploid cytotypes are involved in polyploidy, both lineages of B^7 giving autopolyploids but only one lineage of B^7 producing allopolyploids with either A or B^6 , rendering *Prospero* an excellent system to study genome evolution in polyploids in relation to their parental genomes' divergence. Allopolyploids deviated from strictly additive patterns of parental genomes features, each following a unique evolutionary trajectory. AAB^7B^7 polyploids have lost the paternal 35S rDNA but were otherwise genomically stable, due to strict homologous bivalent pairing. In contrast, $B^6B^6B^7B^7$ allotetraploids exhibited extraordinary variation at all levels of genome organization due to repeated cycles of hybridization. $B^6B^6B^7B^7$ allopolyploids (originally with $2n = 26$) displayed homologous and homoeologous pairing. This resulted in genetically balanced individuals carrying $2n = 25$ to $2n = 28$ chromosomes and the modification of the paternal B^7 genome by massive amplification of the pericentric satellite DNA *PaB6*, fixation of the maternal B^6 -type 5S rDNA loci, and conversion of the 35S rDNA towards the maternal B^6 ribotype. These tetraploids stabilized on $2n = 28$, thus mimicking a B^7 autotetraploid (numerical convergence) and further hybridize with the second B^7 lineage resulting in secondary allotetraploids that can backcross to their parents. This suggests that numerical divergence of parental genomes is insufficient to ensure cytological stability of the polyploid, which instead is achieved via genomic changes.

Key words: allopolyploidy, autopolyploidy, fluorescence and genomic *in situ* hybridization (FISH, GISH), karyotype evolution, *Prospero autumnale* complex.

Introduction

Polyploidy, or whole genome duplication, is one of the most potent forces in the diversification and speciation of plants, particularly in flowering plants (Wendel 2000; Soltis et al. 2009; Weiss-Schneeweiss et al. 2013). Autopolyploidy results from the multiplication of entire chromosome complements, while allopolyploidy involves hybridization and subsequent multiplication of differentiated chromosome sets (Comai 2005; Chen et al. 2007; Otto 2007; Guerra 2008; Weiss-Schneeweiss and Schneeweiss 2013; Weiss-Schneeweiss et al. 2013). Comparative data of the evolution of polyploid genomes are still relatively scarce (Gaeta and Pires 2010; Mandáková et al. 2010; Renny-Byfield et al. 2011; Buggs et al. 2012; Kovařík et al. 2012; Weiss-Schneeweiss et al. 2012, 2013) but emerging patterns suggest that the genomic evolution of polyploids is more dynamic than that of their diploid progenitors (Weiss-Schneeweiss et al. 2013). A variety of patterns of genome evolution have, however, been observed. Thus *Gossypium* and *Spartina anglica* genomes appears to be relatively quiescent with respect to change (Ainouche et al. 2012; Wendel et al. 2012; Gan et al. 2013), while *Nicotiana* (Renny-Byfield et al. 2011; Kovařík et al. 2012), *Tragopogon* (Chester et al. 2012) and wheat (Feldmann et al. 2012) show changes in chromosome complements, genome sizes, and in their repetitive DNA fraction. These changes may affect the parental genomes to differing extents (Koukalova et al. 2010; Malinska et al. 2011; Matsushita et al. 2012; Weiss-Schneeweiss et al. 2012).

The genus *Prospero* (Hyacinthaceae) has two stable species of localized distributions - *P. hanburyi* and *P. obtusifolium* - and a dynamic species complex, *P. autumnale* (L.) Speta, in the Mediterranean region, Europe and western Asia (Parker et al. 1991; Speta 2000; Jang et al. 2013). The *P. autumnale* complex is remarkably variable in chromosome number showing dysploidy, polyploidy and B-chromosomes (Ainsworth et al. 1983; Ebert et al. 1996; Vaughan et al. 1997; Jang et al. 2013), in chromosome structure involving fusions, inversions, translocations, centric shifts and supernumerary chromosomal segments (Taylor

1997; Jang et al. 2013), in genome size (Ebert et al. 1996; Vaughan et al. 1997; Jang et al. 2013; Temsch et al. unpublished data) and repetitive DNA distribution and copy number (Weiss-Schneeweiss et al. unpublished data).

The complex has four diploid cytotypes with unique combinations of basic number, $x = 7$, 6, and 5 genome size, and locations of 5S and 35S rDNA loci and the satellite DNA *PaB6* (Jang et al. 2013; Chapters 1 and 2). Cytotype AA has $x = 7$ and a large genome, and occurs in the western Mediterranean and Portugal. Cytotype B^7B^7 , also with $x = 7$, but with smaller genome size is distributed across the whole Mediterranean basin. Cytotype B^6B^6 with $x = 6$, and intermediate genome size is endemic to Crete, while cytotype B^5B^5 , $x = 5$ and small genome size is endemic to Libya (fig. 1; Ainsworth et al. 1983; Vaughan et al. 1997; Jang et al. 2013). These cytotypes represent phylogenetically well-defined evolutionary lineages (Jang et al. 2013). The widespread cytotype B^7B^7 is paraphyletic and inferred to resemble the ancestral karyotype of the complex (Jang et al. 2013). The $x = 6$ and 5 genomes originated via independent descending dysploidy involving: (i) fusion of chromosomes 6 and 7 to give $x = 6$, and (ii) fusions of chromosomes 1 with 6 or 7, and chromosome 3 and 7 or 6 resulting in $x = 5$ (Jang et al. 2013). Two chromosomally well-defined lineages within B^7B^7 cytotypes differed in type of 5S rDNA locus on chromosome 1 (duplicated vs. single) as well as in the copy and loci number of *PaB6* repeat (Jang et al., Chapter 2).

The diploid cytotypes, with the exception of the phylogenetically youngest B^5B^5 , have given rise to polyploids. Diploid homoploid hybrids are formed in nature between cytotypes B^6 and B^7 (Vaughan et al. 1997; pers. obs.), and some others can be produced via artificial crosses (Jenkins et al. 1988; White et al. 1988; Taylor 1997; Parker unpubl.). Three genomes, A, B^7 and B^6 , participate in allopolyploidization (Ruiz Rejón et al. 1980; Ainsworth et al. 1983; Parker et al. 1991; Ebert 1993; Taylor 1997; Vaughan et al. 1997). These of A and B^7 give AAB^7B^7 , $2n = 4x = 28$, and $AAB^7B^7B^7B^7$, $2n = 6x = 42$, while B^6 and B^7 genomes are reported to form tetraploids with $2n = 26$ (Vaughan et al. 1997). Only cytotype B^7B^7 has

polyploids (Jang et al. 2013), while recently tandemly repeated pericentric satellite DNA *PaB6* has provided further insights into the relationships of the four cytotypes (Jang et al. in prep. – Chapter 2). Phylogenetic relationships of basic diploid cytotypes have also been established (Jang et al. 2013). Thus, chromosomal landmarks and phylogenetic framework are now available for extending our understanding of the complex into polyploids, as has been done in a few other plant genera such as *Tragopogon*, *Nicotiana*, *Melampodium*, *Medicago*, or grasses (Lim et al. 2008; Chester et al. 2012; Kovařík et al. 2012; Rosato et al. 2012; Weiss-Schneeweiss et al. 2012; Mahelka et al. 2013).

The *P. autumnale* complex provides a unique model to explore the evolution of different types of polyploids formed between closely related diploid cytotypes of one species complex. For example, the evolutionary trajectories of two allopolyploids can be followed, whose parental genomes differ in their affinities (AAB^7B^7 and $B^6B^6B^7B^7$). The specific aims of this study are to (1) elucidate evolution of repeats including 5S and 35S rDNA and satellite DNA *PaB6* in auto- and allopolyploids in relation to their parental origin; (2) assess the extent of the variation within polyploid lineages and their dynamics; (3) examine patterns of genome size changes in different polyploid lineages; (4) identify maternal and paternal diploid cytotypes using phylogenetic analyses of plastid and nuclear ITS sequences; (5) assess the stability and interactions of parental genomes of *P. autumnale* in polyploid backgrounds.

Results

Chromosome numbers, genome constitution, and genome size in polyploids

Chromosome numbers and karyotypes were established for all 28 analyzed individuals used in this study (table 1). Each individual represented different population. Genome size has been estimated for all but two individuals due to lack of fresh material.

Table 1. Summary of number of signals of 5S rDNA (in chromosomes 1 [5S¹] and 2 [5S²]), 35S rDNA (in chromosomes 3 [35S³]) and satellite DNA *PaB6* as well as genome size in analyzed individuals of diploids and polyploids of *P. autumnale* complex.

Cytotype	2n	Number of signals of 35S rDNA (in chromosome 3) and 5S rDNA (loci in chromosome 1 and 2) per diploid complement*			cp haplotype/ ITS ribotype**	Number of <i>PaB6</i> signals in pericentric regions	Genome size 1C (pg) ± SD	Deviation from additive value***
<i>Diploids</i>		35S ³	5S ¹	5S ²				
AA	14 ¹	2 (A) [§]	—	2 [§]	A/A		7.85 ± 0.045 [§]	
B ⁷ B ⁷ (1)	14 ¹	2 (B ⁷) [§]	2 (5S ¹ -single) [§]	—	B ⁷ /B ⁷		4.23 ± 0.048 [§]	
B ⁷ B ⁷ (2)	14 ¹	2 (B ⁷) [§]	2 (5S ¹ -dupl) [§]	—	B ⁷ /B ⁷		4.45 ± 0.023 [§]	
B ⁶ B ⁶	12 ¹	2 (B ⁶) [§]	2 [§]	2 [§]	B ⁶ /B ⁶		6.27 ± 0.083 [§]	
B ⁵ B ⁵	10 ¹	2 (B ⁵) [§]	2 [§]	—	B ⁵ /B ⁵		4.86 ± 0.002 [§]	
<i>Polyploids</i>								
AAB⁷B⁷								
H603	28	2 (B ⁷)	2 (B ⁷ ; 5S ¹ -single)	2 (A)	B ⁷ /B ⁷	4 weak, 8-10 very weak	12.70 ± 0.092	+5%
H607	28	2 (B ⁷)	2 (B ⁷ ; 5S ¹ -single)	2 (A)	B ⁷ /B ⁷	4 weak, 8-10 very weak	13.10 ± 0.155	+8.4%
B⁶B⁶B⁷B⁷								
GROUP I								
H153	25	4 (B ^{allo})	4x5S ¹ -single	4x5S ²	B ⁶ /B ⁶	23 strong, 2 medium	12.17	+14.65%
H208	25	4 (B ^{allo})	4x5S ¹ -single	4x5S ²	B ⁶ /B ⁶	22 strong, 3	12.10 ± 0.050	+14.04%

H14	26	4 (B ^{allo})	4x5S ^{1-single}	4x5S ²	B ⁶ /B ⁶	medium 24 strong, 2	11.81 ± 0.373	+11.33%
H96	26	4 (B ^{allo})	4x5S ^{1-single}	4x5S ²	B ⁶ /B ⁶	medium 22 strong, 4	11.67 ± 0.009	+9.94%
H207	27	4 (B ^{allo})	4x5S ^{1-single}	4x5S ²	B ⁶ /B ⁶	medium 25 strong, 2	12.00 ± 0.007	+13.10%
H331	28	4 (B ^{allo})	4x5S ^{1-single}	4x5S ²	B ⁶ /B ⁶ and B ⁷	weaker 26 strong, 2	11.61 ± 0.019	+9.33%
H300	28	4 (B ^{allo})	4x5S ^{1-single}	4x5S ²	B ⁶ /B ⁶	weaker 28 strong	11.53	+8.06%
GROUP II								
H356	28	2 (B ⁷)	2x5S ^{1-single} (B ^{allo}) 2x5S ^{1-dupl} (B ⁷)	2x5S ² (B ^{allo})	B ⁶ /B ⁷	14 strong	9.82 ± 0.249	+0.77%
H363	28	2 (B ⁷), + 1 weak (B ^{allo})	2x5S ^{1-single} (B ^{allo}) 2x5S ^{1-dupl} (B ⁷)	2x5S ² (B ^{allo})	B ⁶ /B ⁷	14 strong	10.25 ± 0.048	+5.12%
H388	28	2 (B ⁷) + 1 weak (B ^{allo})	2x5S ^{1-single} (B ^{allo}) 2x5S ^{1-dupl} (B ⁷)	2x5S ² (B ^{allo})	B ⁶ /B ⁷	14 strong	10.25 ± 0.033	+5.12%
H410	28	2 (B ⁷) + 1 weak (B ^{allo})	2x5S ^{1-single} (B ^{allo}) 2x5S ^{1-dupl} (B ⁷)	2x5S ² (B ^{allo})	B ⁷ /B ⁷	14 strong	10.32 ± 0.064	+5.84%
H434	28	2 (B ⁷)	2x5S ^{1-single} (B ^{allo}) 2x5S ^{1-dupl} (B ⁷)	2x5S ² (B ^{allo})	B ⁷ /B ⁷	14 strong	10.06 ± 0.201	+3.18%

GROUP III H238	28	4 (3 B ^{allo} incl. one weak; 1 B ⁷)	3x5S ^{1-single} (1B ⁷ , 2 B ^{allo}), 1x5S ^{1-dupl} (B ⁷)	3x5S ² (B ^{allo})	B ⁶ /B ⁶ and B ⁷	21 strong	10.84 ± 0.066	+6.48%
GROUP IV H152	28	4 (3B ⁷ , one weak B ^{allo})	3x5S ^{1-single} (B B ^{allo}), 1x5S ^{1-dupl} (B ⁷)	1x5S ² (B ^{allo})	B ⁷ /B ⁷	7 strong	9.25 ± 0.008	+1.64%
H355	28	4 (3B ⁷ , one weak B ^{allo})	3x5S ^{1-single} (1B ⁷ , 2 B ^{allo}), 1x5S ^{1-dupl} (B ⁷)	1x5S ² (B ^{allo})	B ⁷ /B ⁷	7 strong	9.93 ± 0.014	+9.12%
B⁷B⁷B⁷B⁷								
H132	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	8.22 ± 0.035	-2.84%
H172	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	9.14 ± 0.045	+8.02%
H401	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	9.50 ± 0.181	+12.28%
H435	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	9.07 ± 0.028	+7.2%
H534	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	8.53 ± 0.045	+0.83%
H577	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	9.00 ± 0.106	+6.35%
H615	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	9.29 ± 0.008	+9.79%
H628	28	4 (B ⁷)	4x5S ^{1-single}	—	B ⁷ /B ⁷	15-26 weak	-	
H230	28	4 (B ⁷)	4x5S ^{1-dupl}	—	B ⁷ /B ⁷	few weak, 1 strong distal	-	
H310	28	4 (B ⁷)	4x5S ^{1-dupl}	—	B ⁷ /B ⁷	few weak, 2 strong distal	7.45 ± 0.034	-16.3%

* in parentheses genomic origin of the chromosomes carrying rDNA loci identified using the amount and presence of *PaB6*.

**inferred from fig. 6

***Formulas for calculating mean expected parental value of genome size: **AAB⁷B⁷**: (A+B⁷-single)=12.08; **Group I**: (B⁶+ B⁷-single)=10.61; **Group II**: Group I/2 + (B⁷B⁷B⁷B⁷-dupl.)/2=9.75; **Group III**: (Group II)/2 + (Group I)/2=10.18; **Group IV**: (Group II)/2 + (B⁷B⁷B⁷B⁷-single)=9.1; **B⁷B⁷B⁷B⁷-single**: (2x B⁷-single)=8.46; **B⁷B⁷B⁷B⁷-dupl.**: (2x B⁷-dupl.)=8.90.

Abbreviations: A- genome A; B⁷- genome B⁷; B⁶- genome B⁶; B⁵- genome B⁵; 5S^{1-single}: chromosomes 1 with single signal of 5S rDNA; 5S^{1-dupl.}: chromosomes 1 with duplicated signal of 5S rDNA; B^{allo}: rDNA loci originating from Group I (satellite *PaB6* is fully spread therefore more accurate identification of chromosomes of parental genomes is no longer possible).

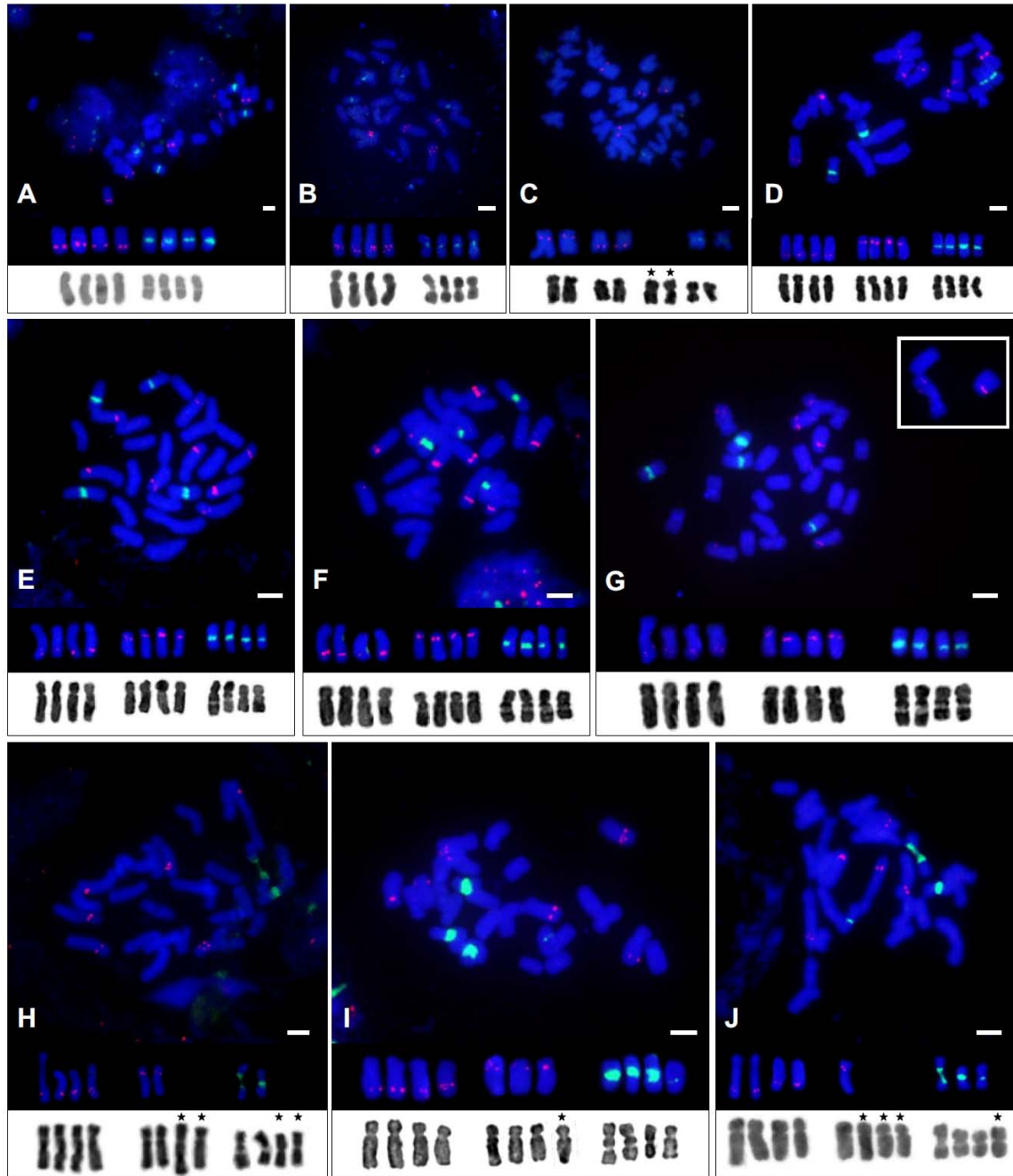


FIG. 2. Localization of 35S (green) and 5S rDNA loci (red) inferred via FISH (fluorescence *in situ* hybridization) in polyploids of *Prospero autumnale* complex. For abbreviations see table 1. (A-B) $B^7B^7B^7B^7$, $2n = 28$: (A) single $5S^1$ locus (H577), (B) duplicated $5S^1$ locus (H310). (C) AAB^7B^7 (H603). (D-J): allotetraploids of B^6 and B^7 origin (D-G) Group I: (D) $2n = 25$ (H153), (E) $2n = 26$ (H14), (F) $2n = 27$ (H207). (G) $2n = 28$ (H331). (H) Group II, $2n = 28$ (H363). (I) Group III, $2n = 28$ (H238). (J) Group IV, $2n = 28$ (H152). Scale bar, 5 μm . Asterisks indicate chromosomes 2 or 3 carrying no 5S or 35S rDNA signals respectively (see supplementary fig. 1; table 1). Inset in (G) shows chromosomes of the same cell which were lying too far from the main chromosome group to be photographed together under high magnification objective. Each individual in cultivation has a unique ID (in brackets, e.g., H577).

Autotetraploids. Autopolyploids have been found exclusively in cytotype B⁷B⁷. Ten analyzed autotetraploid individuals possessed expected $2n = 4x = 28$. The 1C DNA content of autotetraploids varied from 7.45 pg to 9.49 pg (table 1), being usually higher than expected in most individual carrying single 5S rDNA locus in chromosome 1 (5S¹, see below; up to +12.28%; table 1) and lower (−16.3%) in individual carrying duplicated 5S¹ (table 1; fig. 2A–B).

Allotetraploids. Allopolyploids (intercytotype crosses) comprised tetraploids of B⁶ and B⁷ origin ($2n = 4x = 25, 26, 27$, and 28 ; fig. 2D–G), and tetraploids of A and B⁷ origin (AAB⁷B⁷, $2n = 4x = 28$; fig. 2C). Tetraploids of AAB⁷B⁷ constitution possessed uniformly $2n = 28$ chromosomes and the 1C DNA amount of 12.69 or 13.1 pg (table 1) indicating slight genome size increase (5–8.4%) compared to expected additive parental values (table 1). The two parental chromosome complements, A and B⁷, first with 14 larger and the other with 14 smaller chromosomes could be distinguished in the tetraploid cells (fig. 1C; supplementary fig. S1, Supplementary Material online). Only two out of four chromosomes 3 carried NOR (fig. 1C; supplementary fig. S1, Supplementary Material online).

Allopolyploids of B⁶ and B⁷ origin exhibited high levels of variation both in chromosome numbers and in genome size. Chromosome numbers ranged from $2n = 25, 26, 27$, to 28 (fig. 1D–G; supplementary fig. S1, Supplementary Material online). Individuals carrying these numbers were genetically balanced (number of chromosomal arms was constant). This numerical variation resulted from the varying number of submetacentric fusion chromosomes F¹(6–7) of diploid cytotype B⁶B⁶ and corresponding free chromosomes 6 and 7 of cytotype B⁷B⁷. These chromosomes share high degree of homeology pairing either as bivalents or homeologous trivalents in meiosis (figs. 4D–E and 5). Allotetraploids of genomes B⁶ and B⁷ exhibited range of genome sizes from 9.25 pg to 12.17 pg indicating near-additivity of some individuals, but mostly genome size increase up to +14.65% (table 1; fig. 7) in respect to additive parental values. Genome size variation has been structured within four groups of

allopolyploids of B⁶ and B⁷ origin identified on the bases of rDNA and *PaB6* loci distribution patterns (see below).

Patterns of rDNA (5S and 35S rDNA) and *PaB6* satDNA distribution in polyploids

Patterns of 5S and 35S rDNA and satDNA *PaB6* loci distribution were additive compared to expected parental values only in autopolyploids of genome B⁷ (table 1; figs. 2, 3 and 7). Conversely, all allotetraploids experienced changes in the number and localization of rDNA loci/signals compared to additive expected parental values. Despite uniformly pericentric localization of satellite DNA *PaB6*, the unique numbers and size of loci in all diploid cytotypes (see Jang et al. in prep. - Chapter 2; figs. 3 and 7), allowed following its evolution in polyploids. Satellite DNA is highly amplified in cytotype B⁶B⁶ and, to a lesser extent, in cytotype B⁵ (Jang et al. in prep. - Chapter 2), while cytotypes B⁷B⁷ and AA possess much lower copy numbers of this repeat (fig. 7).

Autotetraploids of B⁷ genome (figs. 2A–B, 3A–C, and 7). All ten analyzed autotetraploid B⁷B⁷B⁷B⁷ individuals possessed four interstitial signals of 35S rDNA within the long arm of chromosome 3^{B⁷}, close to centromere. 5S rDNA was located in all four chromosomes 1 (5S¹; fig. 2A–B). Two different patterns of 5S¹ distribution were observed due to presence of either single or duplicated 5S¹ in diploid B⁷ genome (table 1; fig. 7). Eight of ten autotetraploid individuals were homozygous for single 5S¹ signals, while two individuals possessed uniformly duplicated signals of 5S¹ (figs. 2A–B, 7).

Satellite DNA *PaB6* in autopolyploids B⁷B⁷B⁷B⁷ was present in relatively low copy numbers, revealing only weak or very weak signals in pericentric regions of varying number of chromosomes (figs. 3A–C and 7; table 1). Due to signal size, the determination of their exact number was difficult thus their range, rather than absolute signal numbers, is given. Eight individuals homozygous for single 5S¹ signals typically possessed pericentric signals of *PaB6* in 15 to 26 chromosomes (figs. 3A and 7). This number roughly corresponded to the

expected additive pattern of B^7B^7 diploids with single $5S^1$ (fig. 7). Individuals carrying exclusively duplicated $5S^1$ signals had very weak *PaB6* signals in pericentric regions of a few chromosomes only, also corresponding to expected loci number inherited from diploids with duplicated $5S^1$ locus (figs. 3B–C and 7). Both analyzed autopolyploid individuals with duplicated $5S^1$, however, differed from their diploid progenitors in the presence of one or two chromosomes with additional and much stronger, distally located *PaB6* signals (fig. 3B–C). Chromosome(s) carrying the distal signal most likely represent chromosome 2, although chromosome 5 cannot be excluded (supplementary fig. S1, Supplementary Material online).

Allotetraploids of AA B^7B^7 (table 1; figs. 2C, 3D, and 7). Two analyzed individuals of AAB^7B^7 allotetraploids possessed two out of four expected 35S rDNA signals, with consistent loss of two 35S rDNA signals from chromosomes 3^A (chromosome 3 of A genome; figs. 2C and 7). In contrast, 5S rDNA loci number and localization was additive with two interstitial signals of 5S rDNA within the short arm of two chromosomes 2 contributed by genome A ($5S^2$), and two distal signals of 5S rDNA in two chromosomes 1 of B^7 origin ($5S^1$; figs. 2C and 7). The distribution and amount of satellite DNA *PaB6* in AAB^7B^7 chromosomes corresponded to the expected additive values of parental taxa (figs. 3D and 7), with up to four weak and eight to ten very weak signals in pericentric regions of some chromosomes (fig. 3D).

Allotetraploids of B^6 and B^7 genomic origin (figs. 2, 3 and 7). Sixteen allotetraploid individuals of B^6 and B^7 genomic constitution were analyzed. They have been primarily identified on the bases of distinct chromosome numbers ($2n = 25, 26, 27$) or, for individuals with $2n = 28$, numerically mimicking $B^7B^7B^7B^7$ autotetraploids, by the presence of 5S rDNA in chromosomes 2 and amplified *PaB6* signals (both features known only from parental B^6 genome). In addition to variation in chromosome numbers ($2n = 25, 26, 27, 28$; see below and fig. 5) and genome size (table 1), four patterns of 5S rDNA, 35S rDNA and *PaB6* distribution were detected in these individuals:

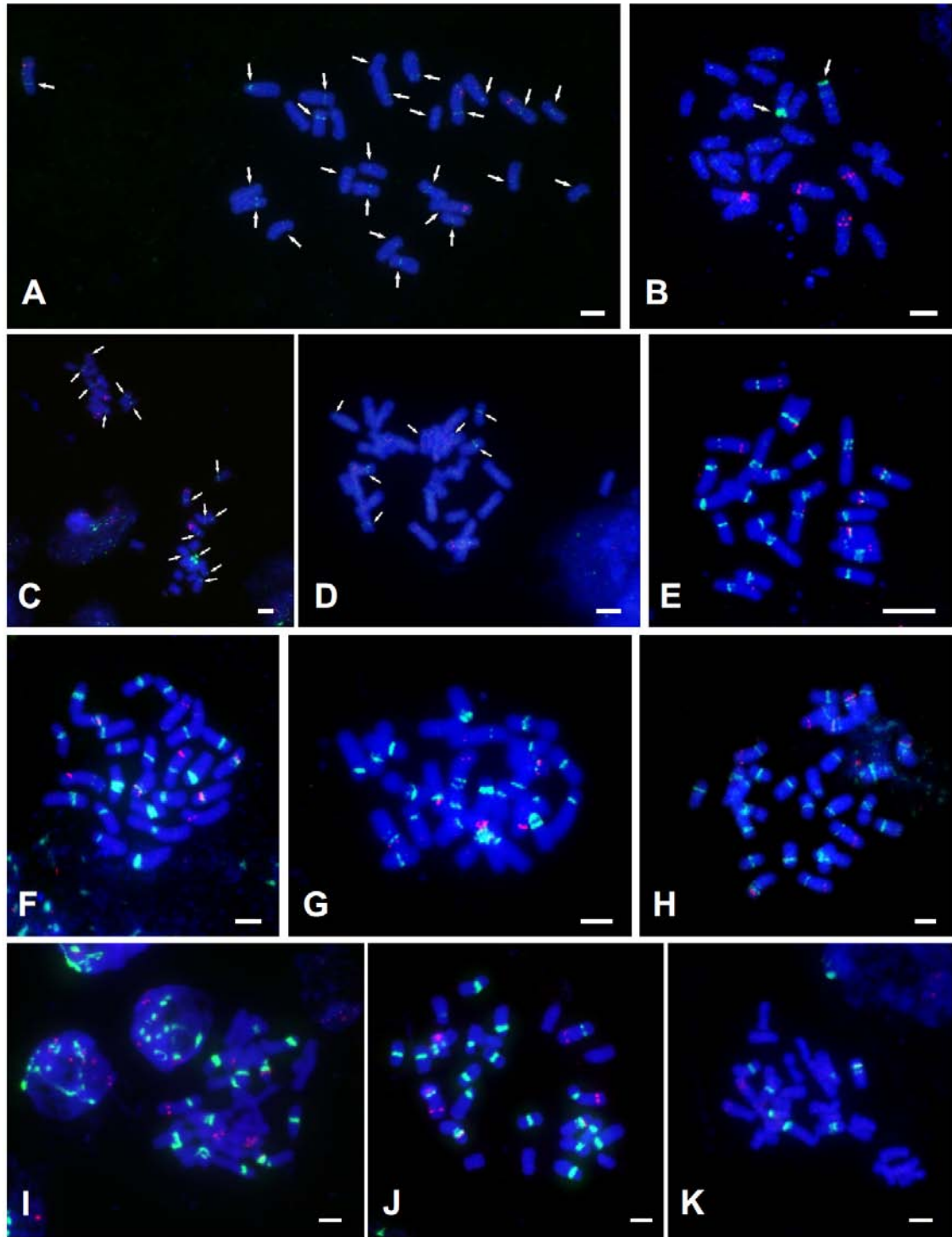


FIG. 3. FISH with 5S rDNA (red) and satellite DNA *PaB6* (green) in diploids and polyploids of *Prospero autumnale* complex. (A-C) $B^7B^7B^7B^7$ all $2n = 28$: (A) single $5S^1$ locus (H534). (B) duplicated $5S^1$ locus (H310). (C) duplicated $5S^1$ locus (H230). (D) AAB^7B^7 (H603). (E-K) $B^6B^6B^7B^7$: (E-H) Group I, *PaB6* amplified in all chromosomes: (E) $2n = 25$ (H153). (F) $2n = 26$ (H14). (G) $2n = 27$ (H207). (H) $2n = 28$ (H331). (I) Group II, 14 strong *PaB6* signals (H363). (J) Group III, 21 strong *PaB6* signals (H238). (K) Group IV, 7 strong *PaB6* signals (H152). Arrows indicate satellite DNA *PaB6* signals. Scale bar, 5 μ m.

Group I (“primary allotetraploids of B⁶ and B⁷ origin”): Allotetraploids with $2n = 25$ (2 individuals), 26 (3 individuals), 27 (1 individual), and $2n = 28$ (2 individuals), despite different diploid chromosome numbers, possessed identical 5S and 35S rDNA loci/signal number and distribution (except for individual H178 with $2n = 26$; supplementary fig. S2G–H, Supplementary Material online). Four interstitial signals of 35S rDNA were detected within the long arms of all four chromosomes 3, four interstitial signals of 5S rDNA ($5S^2$) within the short arms of all four chromosomes 2, and four distally located single 5S rDNA signals ($5S^1$) within the long arms of all four chromosomes 1. While the numbers of 35S and $5S^1$ rDNA signals were additive, only two signals of $5S^2$ were expected (contributed by parental cytotype B⁶B⁶), but instead four signals were observed (figs. 2D–G and 7). Satellite *PaB6* has been detected as strong pericentromeric signals in all chromosomes (figs. 3E–H and 7). Occasionally, two or four pericentric signals were weaker, polymorphism encountered also in diploid B⁶ genome. Allotetraploids with $2n = 25$, 26 and 27 possessed additional weak but consistent *PaB6* signal located interstitially within the long arm of submetacentric fusion chromosome F₁(6–7) (fig. 3E–G). Despite odd chromosome numbers in plants with $2n = 25$ and 27, meiosis was regular with bivalents and occasional trivalents formed between fusion chromosome (6–7) of B⁶ origin and free chromosomes 6 and 7 of B⁷ origin where necessary (fig. 4D–E).

One individual with $2n = 26$ (H178; supplementary fig. S2G–H, Supplementary Material online) deviated from this pattern having: (i) *PaB6* signals in only 18 of 26 chromosomes, (ii) four $35S^3$ signals, two in chromosomes with amplified *PaB6* locus, and two in chromosomes with very weak *PaB6* signal (of B⁷ origin); (ii) four signals of $5S^1$, two in chromosomes 1 with amplified *PaB6* (one with deletion in short arm), one in chromosome 1 with weak and one without satellite DNA *PaB6* signals; (iii) three $5S^2$ rDNA signals in three of four chromosomes 2 coinciding with strong *PaB6* signals (of B⁶ origin). Four other chromosomes did not possess detectable *PaB6* loci and most likely represented one

of each chromosomes 4, 5, 6 and 7. This individual is not included in fig. 7. It could represent aberrant individual (as evidenced by deletion in chromosome 1), or intermediate stage of amplification of *PaB6* repeats in recently formed allotetraploid (supplementary fig. S2G–H, Supplementary Material online).

Groups II, III, IV (“secondary allotetraploids of B⁶ and B⁷ origin”): Eight allotetraploid individuals of B⁶ and B⁷ origin, despite uniform chromosome number of $2n = 28$ (supplementary fig. S1, Supplementary Material online) possessed different and non-additive numbers of rDNA loci and varying numbers of strongly amplified *PaB6* loci. The number of 35S rDNA signals ranged from one to four (expected: four signals in all four chromosomes 3), always located interstitially in chromosomes 3 (figs. 2H–J and 7). 5S rDNA signal numbers ranged from five to eight (expected: six signals for allotetraploids, four for autotetraploids). Locus of 5S¹ rDNA was observed in all chromosomes 1, but was heterozygous in all individuals with two patterns detected: (1) two chromosomes 1 carried single and two duplicated 5S¹ rDNA ($2 \times 5S^{1\text{-single}} + 2 \times 5S^{1\text{-dupl}}$; table 1; figs. 2H–J and 7); (2) there individuals possessed three single and one duplicated 5S¹ rDNA signals ($3 \times 5S^{1\text{-single}} + 1 \times 5S^{1\text{-dupl}}$; supplementary fig. S2G–H, Supplementary Material online). Combination of rDNAs and *PaB6* signals in those allotetraploids allowed their assignment to three further groups (figs. 2H–J, 3I–K, and 7):

Group II: Five individuals carried *PaB6* signals in 14 chromosomes (7 pairs), with remaining seven pairs lacking detectable amounts of *PaB6* (figs. 2H, 3I and 7; table 1). Four 5S¹ rDNA signals were uniformly detected: (i) two single in two chromosomes 1 which carried also strong *PaB6* signals and (ii) two duplicated in two chromosomes 1 lacking detectable *PaB6* signals. 5S² rDNA locus was detected in two chromosomes 2 which also carried strong *PaB6* signals, while two remaining chromosomes 2 lacked both 5S rDNA and *PaB6* signals. 35S rDNA signals were detected in two of four chromosomes 3 which lacked *PaB6* signals (supplementary fig. S2C, Supplementary

Material online; table 1). Additionally, weak 35S rDNA signal in one of two remaining chromosomes 3, both of which carried strong *PaB6* signals, was detected in three of five individuals analyzed (figs. 3I, 7, and supplementary fig. S2B, Supplementary Material online; table 1).

Group III: Two individuals carried *PaB6* signals in seven chromosomes, with twenty-one chromosomes lacking detectable amounts of *PaB6* (figs. 2J, 3K, and 7; table 1). They possessed four 5S¹ rDNA signals: (i) one single 5S¹ signal in chromosome 1 with strong *PaB6*, (ii) two single 5S¹ signals in two chromosomes 1 lacking *PaB6*; (iii) one duplicated 5S¹ signal in chromosome 1 without *PaB6*. Only one of four chromosomes 2 carried 5S² rDNA signal and it also possessed strong *PaB6* signal, while three other chromosomes 2 possessed no detectable *PaB6* signals. Three 35S rDNA signals were detected in three chromosomes 3 which also lacked *PaB6* signals, while one chromosome 3 possessed very weak 35S rDNA and strong *PaB6* signals (supplementary fig. S2F, Supplementary Material online; table 1).

Group IV: One individual (H238) carried *PaB6* signals in 21 of 28 chromosomes (3 sets of chromosomes 1–7), with seven chromosomes lacking detectable amounts of *PaB6* (figs. 2I, 3J, and 7; table 1). Four 5S¹ rDNA signals were observed in four chromosomes 1: (i) one duplicated signal in chromosome 1 which had very weak *PaB6* signal, and (ii) three single 5S¹ signals, two in chromosomes with strong and one with very weak *PaB6* signals (table 1; figs. 3J and 7). Three chromosomes 2 carried 5S² rDNA and strong *PaB6* signals, and one chromosome 2 lacked 5S² rDNA and possessed only very weak *PaB6* signal. Three chromosomes 3 carried 35S rDNA signals, two with strongly amplified *PaB6* signals and one with very weak *PaB6* signal. Fourth chromosome 3 carried very weak 35S rDNA and strong *PaB6* signal (table 1; supplementary fig. S2D–E, Supplementary Material online).

None of the analyzed allotetraploids in *P. autumnale*, neither of A and B⁷, nor of B⁶ and

B⁷ origin, exhibited expected additive patterns of rDNA loci/signals numbers (sum of parental loci and signals) with signal gain prevailing over loss for both 5S rDNA loci and loss prevailing over gain for 35S rDNA locus (fig. 7).

Origin of polyploids inferred from molecular phylogenetic analyses of cpDNA (*ndhA*, *psbD-trnT*, *trnT-trnL*) and nrITS sequences and evolution of 35S rDNA ribotypes

Sequences of three plastid regions and nuclear ITS have been analyzed to infer the relationships and identify diploid parental cytotypes of polyploids. Phylogenetic analyses of ITS regions complemented by FISH data allowed the fate of NOR-chromosomes and 35S rDNA ribotype(s) in the polyploid genomes to be determined. Thus inferences of 35S rDNA conversion, homogenization and loss could be made.

Direct sequencing of ITS regions resulted in single copies of ITS regions for all but two polyploid individuals of B⁶ and B⁷ origin (H238 of group III; H331 of group I) indicating either loss of one of the parental loci or efficient 35S rDNA conversion and homogenization. Cloning of ITS regions has been attempted for selected individuals (not shown) and usually only one type of rDNA was recovered among multiple clones. Cloning of PCR products of individual H238 (Group III) for which direct sequencing was indicative of the presence of both parental copies (supplementary fig. 3, Supplementary Material online) recovered both parental ribotypes.

rDNA conversion, evidenced in polyploids which retained 35S rDNA in all four NOR-chromosomes 3 was directed towards maternal ribotype in Groups I and IV. Five individuals of Group II experienced loss (complete or near-complete) of two NORs of one (and always the same) genome (of Group I) regardless whether it acted as maternal or paternal genome donor (crosses in both directions were documented; table 1).

ITS sequence data of diploid individuals published earlier (Jang et al. 2013) have been included in the analyses (table 1; supplementary table. S1, Supplementary Material online).

Both MP (maximum parsimony) and ML (maximum likelihood) analyses (fig. 6) resulted in topologically similar and well-resolved trees.

ITS sequences of 28 polyploid individuals and 28 diploids, all cytologically characterized and representing all species and cytotypes of the genus *Prospero* with *P. obtusifolium* and *P. hanburyi* as outgroups, have been used for the analyses (fig 6A; table 1; supplementary table. S1, Supplementary Material online). Phylogenetic analyses of ITS region indicated monophyly of *P. autumnale* complex, clear separation of diploid cytotypes AA and B⁶B⁶, and paraphyly of B⁷B⁷. Cytotype B⁵B⁵ was nested within B⁷B⁷ cytotype (fig. 6A). All autotetraploid B⁷B⁷B⁷B⁷ individuals and allotetraploids AAB⁷B⁷ nested within large clade encompassing diploids of B⁷ cytotype (clade I; fig. 6; BS 97/95 MP/ML). Allotetraploids of B⁶ and B⁷ origin were recovered in two clades: (i) all allotetraploid individuals of Group I (satellite *PaB6* amplified strongly in all chromosomes) grouped with diploids of cytotype B⁶B⁶ in clade II (BS 99/97; MP/ML); (ii) five individuals of Group II (14 *PaB6* signals) and two individuals of Group IV (7 *PaB6* signals) were recovered with B⁷ genome in clade I (BS 97/95; MP/ML). Two allotetraploids of B⁶ and B⁷ origin, H238 (Group III; 21 signals of *PaB6*) and H331 (Group I; 28 signals of *PaB6*), possessed both parental 35S rDNA ribotypes and were accordingly recovered both in clade I and II.

Phylogenetic reconstructions based on three plastid DNA regions also clearly separated diploid cytotypes A, B⁷, B⁶ and B⁵, the latter nested within B⁷ (fig. 6B). Autopolyploids of B⁷ cytotype grouped with B⁷B⁷ diploids albeit in two different clades (I and II; fig 6B). Allotetraploids AAB⁷B⁷ were recovered in clade I (BS 96/89). Although AA diploids were also included in this clade, they formed distinct subclade (BS 85/82) indicating that B⁷ genome (similar to the genome that gave rise to B⁶ diploid lineage and likely extant) acted as the maternal parent. Polyploids of B⁶ and B⁷ genomes, grouped either in clade I or II. All individuals of Group I, one individual of Group III (H238; 21 *PaB6* signals) and three individuals of Group II (14 *PaB6* signals) were recovered in a subclade (albeit with low

support) of B^6B^6 . Two remaining individuals of Group II (14 *PaB6* signals) and two individuals of Group IV (7 *PaB6* signals) shared B^7 haplotype as maternal.

Genomic constitution of polyploids and meiotic chromosome pairing

Both types of allopolyploids, AAB^7B^7 and $B^6B^6B^7B^7$ were subjected to analyses of genomic composition and intergenomic exchanges using genomic *in situ* hybridization (fig. 4A–C, F–G). Additionally, diploid homoploid F_1 hybrid of B^6B^7 has been used as a internal control of GISH efficiency. GISH with genomic DNA of AA and B^7B^7 diploid cytotypes resulted in clear parental genomes differentiation in allotetraploids AAB^7B^7 . Genomic AA probe clearly labeled chromosomes 14 chromosomes (7 pairs) and remaining 14 chromosomes were painted with B^7B^7 genomic probe (fig. 4B–C). Two NOR-bearing chromosomes were clearly shown to belong to genome B^7B^7 (as indicated by all other analyses; fig. 4B). Only one small intergenomic exchange has been detected in the analyzed individual (fig. 4B–C). Analyses of meiotic configurations in metaphase I indicated exclusive homologous bivalent pairing (fig. 4A).

GISH with genomic DNA of diploid B^6 and B^7 cytotypes has been applied both to genomically stable diploid homoploid hybrid (B^6B^7 ; see Jang et al., in prep., Chapter 2) and to allotetraploid individual (fig. 4F–G) with $2n = 26$ (Group I). GISH failed to identify parental chromosomes in both polyploid and diploid hybrids, even when high hybridization stringency was used. Some chromosomes of diploid B^6B^7 hybrid showed slightly stronger hybridization with one or the other parental genomic DNAs (B^7) but it was too weak to allow unambiguous identification of parental chromosomes (fig. 4F). The identification of parental chromosomes sets in diploid hybrid was possible mostly due to differences of *PaB6* signal strength in parental genomes. Allopolyploid experienced massive amplification of *PaB6* signals in all chromosomes, which it obscured other genomic signals, if any (fig. 4G). Attempts to block *PaB6* hybridization in polyploids were so far unsuccessful. Analysis of

meiotic configurations of allotetraploid of B⁶ and B⁷ origin ($2n = 25$; Group I; fig. 4D–E) using FISH with *PaB6* probe indicated regular bivalent pairing, with only one trivalent (fusion chromosome F¹(6–7) with two free chromosomes 6 and 7). Due to GISH failure, the extent of homologous and homeologous bivalent pairing could not be estimated.

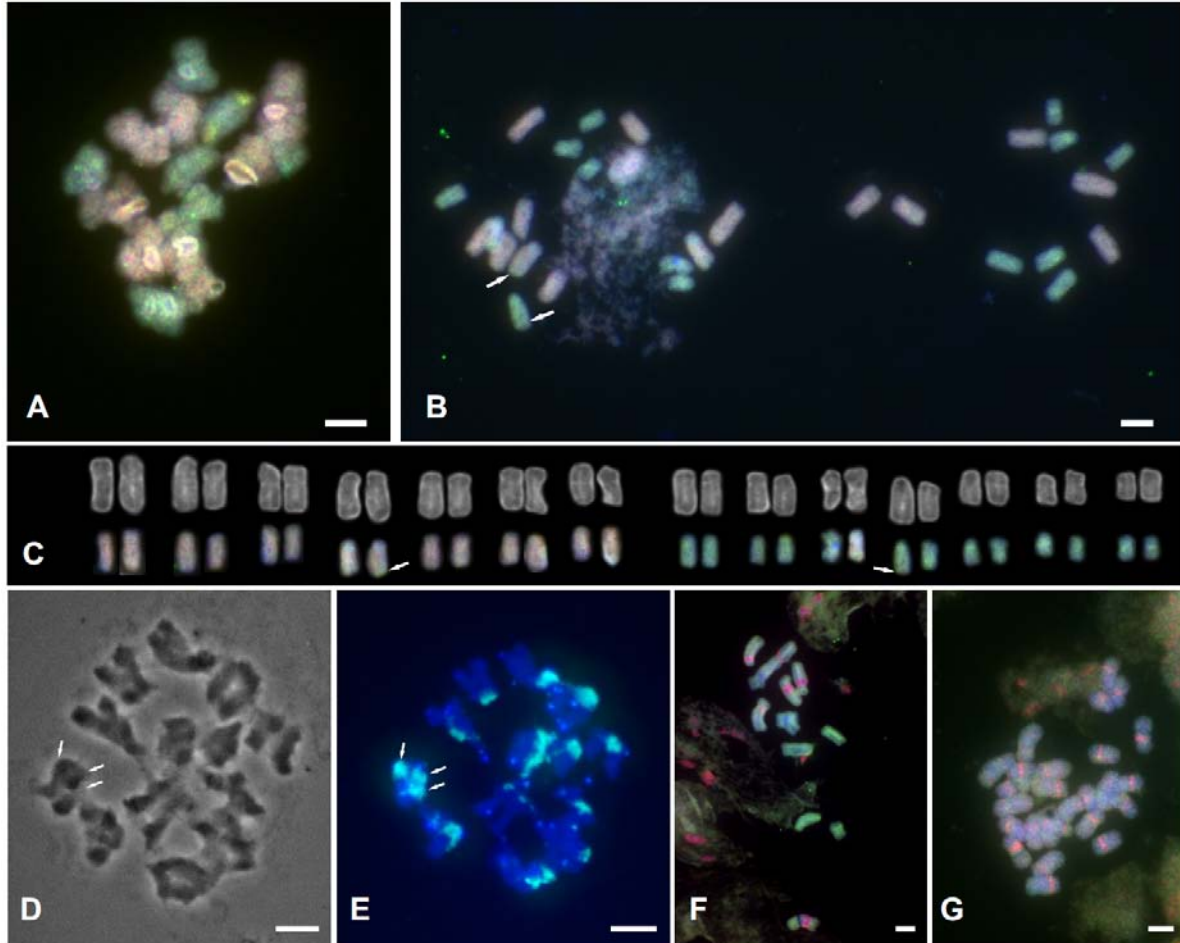


FIG. 4. FISH with satellite DNA *PaB6* (green) and GISH (genomic *in situ* hybridization) in allopolyploids of *Prospero autumnale* complex. (A–C) localization of A (red) and B⁷ (green) genomic DNA in allotetraploid AAB⁷B⁷. (A) Meiotic metaphase I showing 7 homologous bivalents from A genome, 7 homologous bivalents from B⁷ genome and 35S rDNA in yellow, H606. (B, C) Mitotic chromosomes, 14 of which are labelled with DNA of genome A and 14 with genome B⁷ and cut-out karyotype of the same cell photographed in phase contrast (upper row) and after GISH (lower row; H603). Arrows in (B–C) indicate small intergenomic exchanges. (D–E) Meiotic metaphase I of the B⁶B⁶B⁷B⁷ tetraploid ($2n = 25$) with 12 bivalents and 1 trivalent (H153). Arrows indicate trivalent which contains strong pericentromeric satellite DNA *PaB6* signals. (F–G) Mitotic metaphase chromosomes after GISH with genomic DNA of B⁶ (red) and B⁷ (green) signals: (F) diploid homoploid hybrid B⁶B⁷ ($2n = 13$; H364), (G) allotetraploid ($2n = 28$; H331). Scale bar, 5 μ m.

Discussion

Polyploidization contributes to diversification and evolution of most of plant groups (Wendel 2000; Soltis et al. 2009; Weiss-Schneeweiss et al. 2013). Polyploidy has previously been also reported in *Prospero* (Ainsworth et al. 1983; Ebert 1993; Vaughan et al. 1993, 1997; Taylor 1997; Speta 2000; Hamouche et al. 2010), but exclusively within *P. autumnale* complex, in contrast to the two other species of the genus, *P. obtusifolium* and *P. hanburyi* known only as diploids (Speta 1986; Ebert 1993; Vaughan et al. 1997; Hamouche et al. 2010). All diploid cytotypes of *P. autumnale* complex participate in polyploidization with the exception of the phylogenetically most-derived cytotype B⁵B⁵ endemic to Libya. Autopolyploids are formed only within cytotype B⁷B⁷, but within both of its lineages, while two genomic types of allopolyploids exist originating from hybridization between either cytotypes AA and B⁷B⁷ or cytotypes B⁶B⁶ and B⁷B⁷ (Ainsworth et al. 1983; Parker et al. 1991; Vaughan et al. 1997). This study reports in-depth comparative analyses of genomic and chromosomal evolution of tetraploids of different origins in *Prospero autumnale* complex.

Origin and dynamics of evolution of polyploids in genus *Prospero*

Identification of tetraploids in *Prospero autumnale* based on their chromosome numbers is trivial but establishing their specific type and parental origin is not an easy task (fig. 7) especially in the absence of their morphological differentiation. Tetraploid plants with $2n = 28$ and distinct two sets of chromosomes, one large and the other smaller, occurring in western Mediterranean are relatively easy to identify as allotetraploids of genomes A and B⁷. These allotetraploids have very stable genomes, and hardly any variation has been detected previously and in the present study (Ainsworth et al. 1983; Parker et al. 1991; Taylor 1997; Vaughan et al. 1993, 1997), except for the occasional presence of B chromosomes and supernumerary chromosomal segments. Analyses of meiotic chromosome pairing in these

polyploids indicated the presence of bivalents in metaphase I, despite formation of multivalent in zygotene which are resolved before the onset of metaphase (Jenkins et al. 1988; White et al. 1988). Homologous bivalent pairing has also been observed in the current study, and demonstrated using GISH. Allotetraploids AAB^7B^7 do not experience any significant changes and their genomes largely represent the sum of parental genomes with diploids B^7B^7 recovered as maternal and AA as paternal parents. Following genome merger, 35S rDNA loci are lost from paternal genome, as also shown earlier (Vaughan et al. 1993). Neither 5S rDNA loci nor *PaB6* repeats experience any significant changes. It is likely that the genomic divergence of the parental cytotypes might be sufficient, as evidenced e.g., by difference in genome sizes, to prevent or at least limit intergenomic interactions in polyploid. It has been hypothesized that cytotype AA has been isolated during glaciations from widespread and most likely ancestral B^7 cytotype in westernmost Mediterranean. This isolation might have allowed the accumulation of sufficient genomic differences to create distinct genomic entity. Once secondary contact was established, AA and B^7B^7 cytotypes hybridized and produced AAB^7B^7 allotetraploids. Present-day A and B^7 diploids are the only ones that do not cross in nature or in garden experiments which lends further support for their genomic distinctiveness.

Autotetraploids ($2n = 28$) of B^7 genomes are widespread in northern, but also in southeastern part of the distribution range of *Prospero*, but extend their distribution range beyond that of their progenitor diploid cytotype. They carry only one 5S rDNA locus in chromosome 1, similar to their diploid progenitors. The existence of two lineages in diploid B^7B^7 cytotype (Jang et al. 2013) is also reflected in their autopolyploids. These two lineages differ in the presence of either single or duplicated 5S¹ rDNA locus (5S rDNA in chromosome 1) and corresponding differences in the number of copies and signals of *PaB6* satellite DNA in the pericentromeric regions of chromosomes. Each of the two diploid lineages produces own autotetraploids which carry exclusively single or exclusively duplicated 5S¹ rDNA, and

accordingly higher or lower *PaB6* copy numbers. Interestingly, genome size of plants with single loci is near-additive or higher than expected sum of parental values, while genome downsizing has been observed in individual with duplicated 5S¹ rDNA locus. Unexpectedly, autotetraploids with duplicated 5S¹ rDNA locus carry one or two larger and subterminally located loci of *PaB6* absent from their diploid progenitors and from other autopolyploids. What is the nature of this additional signals is currently unknown, but it is not likely that it represents supernumerary chromosomal segments which are usually detected as length differences of the homologous chromosomes (Parker et al. 1991; Taylor 1997). Phylogenetic analyses of nrITS and plastid sequences did not assign any evolutionary significance to 5S¹ rDNA duplication, but phylogenetic analyses of 5S rDNA spacer (Emadzade et al., unpublished data) indicated that this duplication might be phylogenetically and evolutionarily informative (Jang et al. 2013 - Chapter 1; Emadzade et al., in prep.). Thus, such divergence might represent early stage of the evolution of new cytotypes within *P. autumnale* complex.

Last class of tetraploids represents allotetraploids of B⁶ and B⁷ genomes ($2n = 25-28$). All these plants occur on Crete, sympatrically with one of their parental cytotypes, B⁶B⁶, endemic to this island. Due to numerical convergence in these allotetraploids, some of which mimic autopolyploids in chromosome numbers ($2n = 28$) it is difficult to establish their exact genomic affinity using chromosome numbers alone. Variation in chromosome numbers is only apparent in what we call Group I or “primary allotetraploids of B⁶ and B⁷” (figs. 5 and 7; table 1) originating as crosses of B⁶ cytotype acting as maternal genome donor and B⁷ within single 5S¹ rDNA locus as paternal. The numerical variation stems from homo- and homeologous pairing of the chromosomes in meiosis. All chromosomes in these polyploids, regardless of the diploid chromosome number, pair strictly as bivalents, except for large submetacentric fusion chromosome F₁(6–7) of B⁶ origin which can form trivalents with its counterparts in genome B⁷, free chromosomes 6 and 7. Combination of homologous and homoeologous chromosome pairing of the latter three chromosome types, and most likely

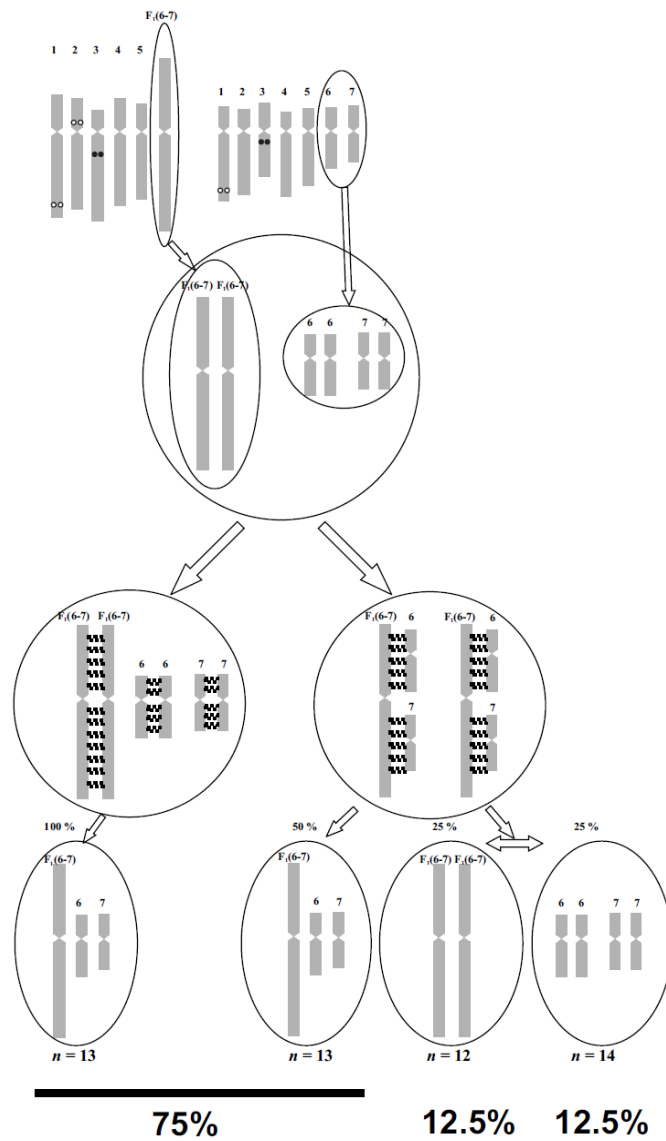
also of others, in original $2n = 26$ F₁ plants, gives rise to chromosomally unique but balanced mosaic gametes with three different chromosome numbers: $n = 12$, 13 , and 14 (but constant chromosome arms number). These gametes are produced with different frequencies, with most common $n = 13$. Free joining of the gametes produces next generation of plants varying in chromosome numbers from $2n = 24$ to $2n = 28$, albeit having constant number of chromosomal arms (Stebbins 1971). Allotetraploid individuals with $2n = 25$, 26 , 27 , and 28 were found in this and previous studies (Taylor 1997), while plants with $2n = 24$ have never been reported. The numerical variation is accompanied by changes in genome composition. 5S² rDNA locus spreads from two chromosomes 2 of B⁶ origin to two chromosomes 2 of B⁷ origin. *PaB6* repeats experience amplification in pericentric loci of all chromosomes. All analyzed individuals of Group I possessed already amplified *PaB6* signals and additional 5S rDNA signals, except for one (H178; $2n = 26$) which possessed only 18 strong *PaB6* signals. This individual might represent earlier stages of incomplete *PaB6* amplification. The current data suggest that the mechanism of satellite DNA *PaB6* spread and amplification in all chromosomes of B⁷ origin might be related to homoeologous chromosome pairing and recombination. *PaB6* present in high copy numbers in all chromosomes of B⁶ origin spans large pericentric regions extending to interstitial regions of chromosomes. Unlike centromere, these regions might participate in recombination and facilitate spread of *PaB6* in one single step following homeologous pairing. Such amplification might also be mediated by recombination-induced extrachromosomal circular DNA (eccDNA; Navrátilová et al. 2008; Cohen et al. 2010; Jang et al., Chapter 2).

High levels of amplification of *PaB6* in B chromosomes of *Prospero* which do not recombine with standard chromosome complements (Jang et al., Chapter 4) lends support at least to the presence of eccDNAs containing *PaB6* monomers. 5S rDNA spread to all four chromosomes 2 might be facilitated by co-amplification with *PaB6* repeats or result from homoeologous recombination. Locus of 5S² rDNA, unlike 5S¹ rDNA locus on chromosome 1,

is located in pericentric region of chromosome 2 adjacent to *PaB6* locus. Diploid homoploid hybrids of genomes B⁶ and B⁷ are formed *de novo* in mixed populations (Jang et al., Chapter 2) and can also be resynthesized in common garden experiments. Meiotic configurations observed in meiosis of these hybrids represent six homoeologous bivalents and one trivalent (Taylor 1997). Analyses of next generations of these hybrids will allow testing of existing hypotheses concerning mechanisms of *PaB6* and 5S rDNA copy number increase.

Despite impressive numerical chromosomal variation of primary B⁶ and B⁷ allotetraploids, just one of these seems to be involved in further hybridization. Only individuals with $2n = 28$ of Group 1 were inferred to successfully hybridize with another lineage of B⁷ cytotype which possesses duplicated 5S¹ rDNA locus (fig. 7). These allotetraploids with $2n = 28$ and 14 chromosomes carrying *PaB6* loci would be expected to form homologous bivalents in meiosis. Analyses of meiotic behavior of these plants have been initiated. Polyploids of this composition might correspond to plants reported earlier by Vaughan et al. (1997) as autopolyploids of B⁷, albeit possessing two different classes of chromosomes differing in size (smaller chromosome complement referred to as diploid C-genome present only in polyploids). Three further individuals of B⁶ and B⁷ allotetraploids deviated in patterns of repeat localization from those of Groups I and II. They have been proposed to represent backcrosses of Group II individuals to either primary allotetraploids of Group I (Group III) or B⁷ genome with single 5S¹ rDNA locus (paternal genome of Group I; Group IV; fig. 7). It is likely that these polyploids might be unstable.

(FIG. 5, see next page). Model of chromosomal numerical variation resulting from meiotic homologous and homoeologous chromosome pairing in allotetraploids between B⁶ and B⁷ genomes (Group I) within *Prospero autumnale* complex. From the second row on only pairing and segregation of chromosomes 6 and 7 and fusion chromosome F₁(6-7) are indicated (Jang et al., 2013), because only these cause the variation of chromosome numbers in the gametes (rows 3 and 4). Table below provides overview of crossing possibilities of gametes and the frequencies of newly formed individuals, assuming Mendelian free joining of the gametes.



$$2n = 28, 27, 26, 25, 24$$

$$1 : 12 : 38 : 12 : 1$$

	$6 + 7 + 6 + 7$ 1x	$F_1(6-7) + 6 + 7$ 6x	$F_1(6-7) + F_1(6-7)$ 1x
$6 + 7 + 6 + 7$ 1x	$2n = 28$ 1	$2n = 27$ 6	$2n = 26$ 1
$F_1(6-7) + 6 + 7$ 6x	$2n = 27$ 6	$2n = 26$ 36	$2n = 25$ 6
$F_1(6-7) + F_1(6-7)$ 1x	$2n = 26$ 1	$2n = 25$ 6	$2n = 24$ 1

FIG. 5.

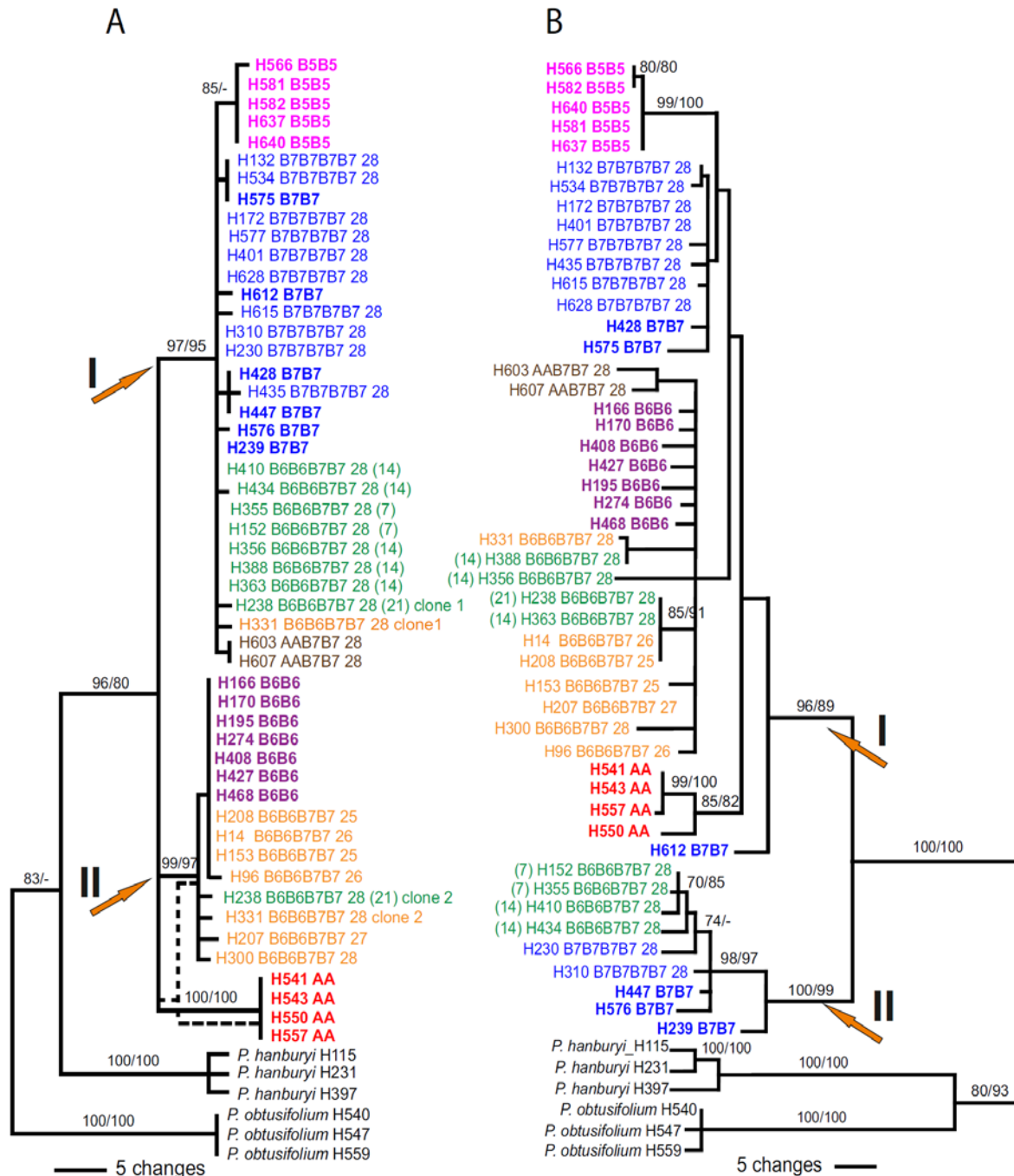


FIG. 6. Phylogenetic relationships of diploids and polyploids of the *Prospero autumnale* complex inferred from ITS (A) and plastid (B) sequence data. Bootstrap support (BS) given above branches as MP/ML (maximum parsimony/maximum likelihood). Arrows indicate major clades (see text for details). Diploids are marked bold (blue, B⁷B⁷; violet, B⁶B⁶; red, AA; pink, B⁵B⁵) with collection number of individual, genome composition, chromosome number (2n) and number of PaB6 signals in brackets, where applicable (see Table S1 and Table 1 for details). Allopolyploids of AAB⁷B⁷ indicated in brown, allopolyploids of B⁶ and B⁷ in orange (Group I) or green (Groups II-IV), autotetraploids B⁷B⁷B⁷B⁷ in blue.

Differences in chromosome numbers of diploid parental genomes seem to be of little importance for polyploid genome stability, at least in *Prospero*. Instead, extent of genomic differentiation might play the most important role. All patterns outlined here suggest that the evolutionary importance of numerical variation in B⁶ and B⁷ polyploids in *Prospero* a byproduct of, extensive genome modifications in primary allopolyploids of B⁶ and B⁷ genomes via homoeologous chromosome pairing. Some evidence of remodeling and homogenization of both sets of parental chromosomes via amplification and spread of pericentric satellite DNA repeats *PaB6* and 5S rDNA in chromosome 2 is presented here. Thus, it is likely, that other changes participating in genome reorganization are also introduced as a result of homoeologous chromosome pairing. Established primary allotetraploids might represent novel genomes sufficiently different from either of the parents, to enable formation of genomically stable and evolutionary more successful allotetraploids via subsequent hybridization to either of the parents or closely related other lineages. Success of these new polyploids would be measured by the homologous rather than homeologous bivalent pairing. Hence, the original phase of genomic instability might be beneficial for overall long-term evolutionary success of polyploids in a group of closely related taxa. Whether such phenomenon is also present in other plant groups remains to be tested.

Evolution of 35S rDNA in polyploids

35S rDNA sites have often been reported to be more variable in number than those of 5S rDNA sites (Moscone et al. 1999; Mishima et al. 2002; Datson et al. 2006; Książczyk et al. 2010; Sousa et al. 2011). Genus *Prospero* and several other plant groups exhibit, however the opposite pattern with 5S rDNA being more variable (Hasterok et al. 2006; Fukushima et al. 2011; Mlinarec et al. 2012a; Weiss-Schneeweiss et al. 2013). 35S rDNA are localized in NORs in one-to-many chromosome pairs per genome (Małuszyńska et al. 1998; Weiss-

Schneeweiss and Schneeweiss 2013). 35S rDNA loci in allopolyploids often experience either complete loss or conversion of repeat types towards one of the parental genomes or towards novel ribotypes (Weiss-Schneeweiss et al. 2013) affecting either paternal or maternal genomes (Skalická et al. 2003, 2005; Guggisberg et al. 2008; Renny-Byfield et al. 2011; Mlinarec et al. 2012b; Weiss-Schneeweiss et al. 2012).

A tightly knit group of polyploids in *Prospero autumnale* complex provides wealth of comparative data on the evolution of 35S rDNA. Two emerging correlations are particularly interesting: (i) 35S rDNA conversion (either towards maternal or paternal ribotype) has been suggested for polyploids whose genomes are still in flux. (ii) 35S rDNA loss has been associated with significant parental genome divergence, in other words, with stable, homologous bivalent pairing allopolyploids (AAB⁷B⁷ and secondary allotetraploids of B⁶ and B⁷ origin; Group II). rDNA copies loss seems to be preceded by ribotype conversion. In contrast to 35S rDNA, 5S rDNA loci in *Prospero* polyploids experienced predominantly increase in signal and copy number or remained additive, and did not undergo any conversion (Emadzade et al., unpublished data).

(FIG. 7, see next page). Each diploid genome is represented by standard ideogram with 5S rDNA (red), 35S rDNA (green) and Satellite DNA *PaB6* in blue. Chromosomes of B⁷ genomes in grey (two diploid lineages and two corresponding autotetraploids), A chromosomes in orange, and B⁶ chromosomes in blue. B⁵ genome does not participate in polyploid formation and was not included. Allotetraploids of B⁶ and B⁷ origin of Group I (primary allopolyploids) indicated in violet because parental chromosomes cannot be distinguished due to amplification of *PaB6* in all chromosomes and spread of 5S² to all chromosomes 2. Arrows indicate directions of crosses; inferred maternal and paternal parents are indicated (based on phylogenetic analyses; Fig. 6).

Conclusions

Comparative data of the evolution of polyploid genomes are still relatively scarce (Mandáková et al. 2010; Renny-Byfield et al. 2011; Buggs et al. 2012; Kovařík et al. 2012) but genomic evolution in polyploids is accepted often to be more dynamic than that of their diploid counterparts (Weiss-Schneeweiss et al. 2007, 2013). Various patterns of polyploid genome evolution have been observed, from relative stability of polyploid genomes (Ainouche et al. 2012; Wendel et al. 2012), to changes on all levels of genome organization (Renny-Byfield et al. 2011; Chester et al. 2012; Kovařík et al. 2012; Gan et al. 2013). *Prospero autumnale* complex encompasses polyploids of all these types exhibiting varying rates of genomic evolution. The stability and evolution of allopolyploid genomes have been suggested to depend on parental divergence (Paun et al. 2009), with polyploid hybrids formed with higher frequency between more divergent parental diploids (Chapman and Burke 2007). Thus, *Prospero* offers an exceptional system in which to study the evolutionary fates of polyploid genomes in relation to their parental divergence (as measured by phylogenetic distance, chromosomal and genomic divergence). A system of closely related diploids creating such diversity on polyploid level is unique in *Prospero*.

Materials and Methods

Plant material

Diploid and polyploid cytotypes of the *Prospero autumnale* complex were cultivated in the Botanical Garden of the University of Vienna (table 1; supplementary table S1, Supplementary Material online). Each individual used for analyses had to be karyotyped due to high level of polymorphisms in the complex. Chromosome numbers and karyotypes were assessed by standard Feulgen staining of meristematic root cells (Jang et al. 2013). Actively growing root-tips were pretreated with 0.05% colchicine for 4h at room temperature, fixed in

ethanol : acetic acid (3 : 1), and stored at -20°C until use (Jang et al. 2013). Anthers in young flower buds used for meiotic analyses were after fixed in ethanol : chloroform : acetic acid (6 : 3 : 1) and stored at -20°C .

Karyotypes were assembled in Corel Photo-Paint X5 (supplementary fig. 1, Supplementary Material online) and idiograms based on at least three randomly selected well-spread metaphase plates per individual (not shown) were constructed. Idiograms of each polyploid cytotype based on 5S and 35S rDNA and *PaB6* satellite DNA FISH signals (see below) have been constructed with the program Autoidiogram (Weiss-Schneeweiss et al. 2007).

Fluorescence *in situ* hybridization (FISH), and genomic *in situ* hybridization (GISH).

Chromosomes for FISH and GISH were prepared by enzymatic digestion and squashing as described in Jang et al (2013). Briefly, meristems were digested with 1% cellulose Onozuka (Serva, Heidelberg, Germany), 1% cytohelicase (Sigma-Aldrich, Vienna, Austria), and 1% pectolyase (Sigma-Aldrich, Vienna, Austria), and squashed in 60% acetic acid. Cover slips were removed at -80°C and preparations air-dried.

Probes used for FISH were: monomer of satellite DNA *PaB6* (Jang *et al.* in prep. – Chapter 2) isolated from the B^6 genome in *P. autumnale* complex in plasmid pGEM-T easy, 35S rDNA (18S/25S rDNA) from *Arabidopsis thaliana* in plasmid pSK+, and genic region of 5S rDNA from *Melampodium montanum* in plasmid pGEM-T easy. Probes were labeled with biotin or digoxigenin (Roche, Vienna, Austria) by PCR (5S rDNA and satellite DNA *PaB6*) or using a nick translation kit (35S rDNA; Roche, Vienna, Austria). Digoxigenin was detected with antidigoxigenin conjugated with FITC ($5\text{ }\mu\text{g mL}^{-1}$; Roche, Vienna, Austria) and biotin with ExtrAvidin conjugated with Cy3 ($2\text{ }\mu\text{g mL}^{-1}$; Sigma-Aldrich, Vienna, Austria; Jang et al. 2013).

Total genomic DNA from diploid cytotypes AA, B^6B^6 , and B^7B^7 was isolated using

CTAB method (Doyle and Doyle 1987; Jang et al. 2013), sheared at 98°C for 5 min, and labeled with digoxigenin or biotin using a nick translation kit (Roche, Vienna, Austria).

GISH was carried out following Schwarzacher and Heslop-Harrison (2000) with modifications. The hybridization mix included 10% (w/v) dextran sulfate, 0.07×SSC, 1% (w/v) SS (salmon sperm DNA) and c. 125 µg mL⁻¹ of each genomic probe. After hybridization, slides were washed three times in 2×SSC at 42°C. Probes were detected using 5µg mL⁻¹ antidigoxigenin conjugated with FITC (digoxigenin), or 2µg mL⁻¹ ExtraAvidin conjugated with Cy3 (biotin) in 2×SSC containing 5% (w/v) bovine serum albumin. Chromosomal DNA was counterstained with 8µg mL⁻¹ DAPI (4',6-diamidino-2-phenylindole) in 2×SSC, and mounted in Vectashield antifade medium (Vector Laboratories, Burlingame, CA, USA).

Chromosomes were analyzed with an AxioImager M2 epifluorescent microscope (Carl Zeiss, Vienna, Austria), images acquired with a CCD camera, and files processed using AxioVision ver. 4.8 (Carl Zeiss, Vienna, Austria) with only those functions that apply to all images equally. A minimum of 20 well-spread metaphases and prometaphases were analyzed in each individual.

DNA amplification, sequencing and phylogenetic approach

Total genomic DNA was extracted from silica gel-dried leaf material as described in Jang et al (2013). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA was amplified and sequenced using universal ITS primers (ITS 18 s F and ITS 26 s R) following the protocol of Jang et al (2013). Three plastid regions were amplified using primers and protocols of Shaw et al (2007; *ndhA*, *psbD-trnT*) and Demesure et al (1995; *trnD-trnT*). All ITS and plastid sequences are deposited in GenBank (accession numbers will be provided in supplementary table S1, Supplementary Material online) and the alignment and trees are deposited in treeBASE (in prep.).

PCR products were checked on 1% (w/v) agarose gel, cleaned with exonuclease I (Exo I) and calf intestine alkaline phosphatase (CIAP; Fermentas, St. Leon-Rot, Germany), and directly sequenced using the PCR primers and dye terminator chemistry (Life Technologies, Vienna, Austria) on a 48-capillary sequencer (3730 DNA Analyzer, Life Technologies, Vienna, Austria). Sequences were assembled in SeqManII (Lasergene, Madison, WI) and manually aligned in BioEdit software ver. 7.0.5.3 (Hall 1999). Three analyzed plastid regions were concatenated for the analyses. Indels were coded as binary characters following the “modified complex coding method” using SeqState version 1.36 (Müller 2005), and the dataset with coded gaps was used in all analyses. A heuristic search for most parsimonious (MP) trees was performed using PAUP 4.0.b10 (Swofford 2002). The analyses involved 1000 replicates of random sequence addition, with tree bisection–reconnection (TBR) branch swapping, saving no more than 10 trees per replicate. All characters were equally weighted and treated as unordered. Strict consensus trees were computed from all equally most parsimonious trees. Nodal support was assessed via bootstrapping (BS; Felsenstein 1985) in PAUP* 4.0b10 with 10,000 bootstrap replicates, each with 10 random sequence addition replicates holding maximally 10 trees per replicate, SPR branch swapping, and MulTrees on.

Maximum likelihood (ML) analyses were conducted for ITS and concatenated three plastid regions using raxmlGUI 1.3 (Silvestro and Michalak 2010) with the GTR+GAMMA nucleotide substitution model. The ML tree and BS for each region were obtained using the rapid bootstrap algorithm (Stamatakis et al. 2008) with 1000 replicates.

Genome size estimation

Genome size of 26 polyploid individuals (leaf material for two individuals was not available) of the *Prospero autumnale* complex was determined by flow cytometry with *Pisum sativum* ‘Kleine Rheinländerin’ (1C = 4.42 pg/1C, Greilhuber and Ebert 1994; Temsch et al. 2010; Jang et al. 2013) as internal standards. Fresh material and the standard were co-chopped in

Otto's buffer I (Otto et al. 1981), filtered through a nylon mesh, and incubated with RNase at 37°C in a water-bath for 30 min. Propidium iodide (PI) staining was performed in Otto's buffer II at 4°C. Each individual (except for two) was measured three times. Measurements were done with a CyFlow ML flow cytometer (Partec, Muenster, Germany) equipped with a mercury arc lamp or a green laser (100mW, 532nm, Cobolt, Sweden) and 1C values were calculated according to the assumed linear fluorescence intensity relationship of both object and standard nuclei (Temsch et al. 2010; Jang et al. 2013). Genome size of diploid cytotypes has been reported earlier (Jang et al. 2013). CVs of all measurements were usually lower than 5% (Greilhuber et al. 2007), and never exceeded 10%.

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Supplementary Material

Supplementary Figure 1: Representative karyotypes of all types of auto- and allopolyploids in *Prospero autumnale* complex analyzed in this study. See Table 1. Scale bar, 5 μ m.

$B^7B^7B^7B^7$ ($2n = 28$): H534



$B^7B^7B^7B^7$ ($2n = 28$): H310



AAB^7B^7 ($2n = 28$): H603



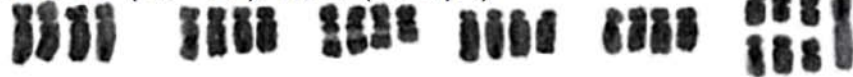
$B^6B^6B^7B^7$ ($2n = 25$): H208 (Group I)



$B^6B^6B^7B^7$ ($2n = 26$): H96 (Group I)



$B^6B^6B^7B^7$ ($2n = 27$): H207 (Group I)



$B^6B^6B^7B^7$ ($2n = 28$): H331 (Group I)



$B^6B^6B^7B^7$ ($2n = 28$): H434 (Group II)



$B^6B^6B^7B^7$ ($2n = 28$): H363 (Group II)



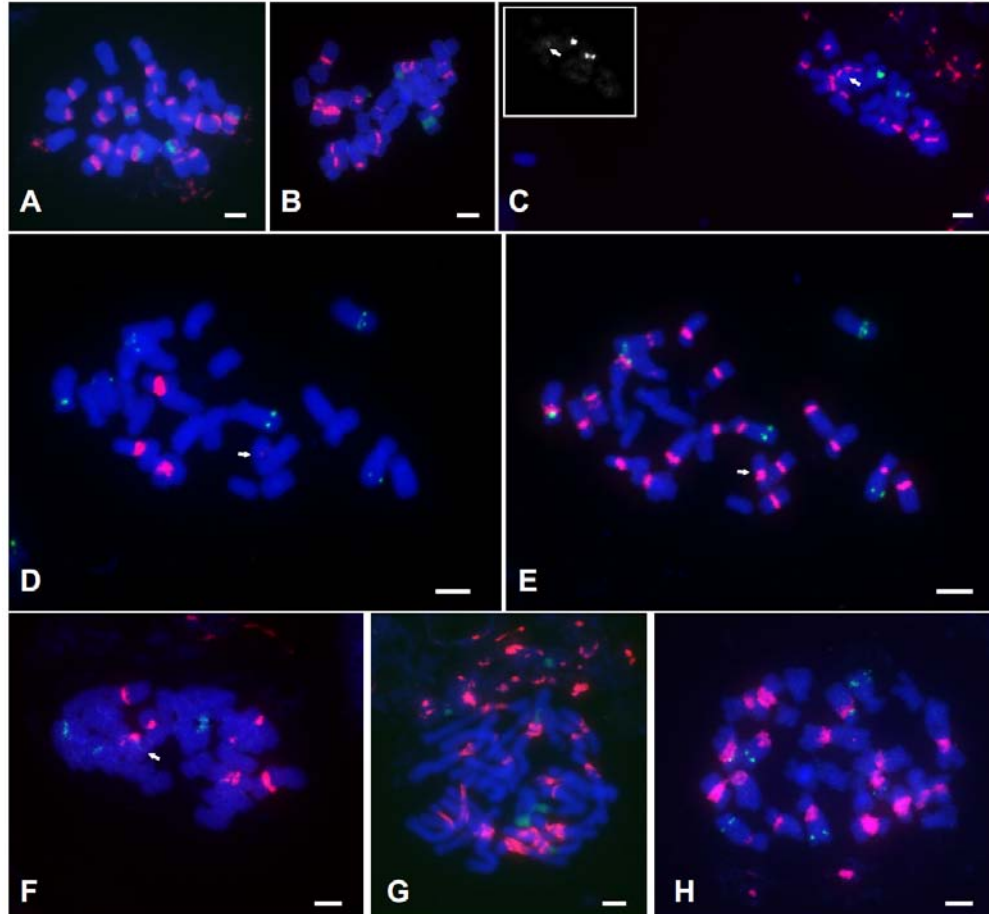
$B^6B^6B^7B^7$ ($2n = 28$): H238 (Group III)



$B^6B^6B^7B^7$ ($2n = 28$): H152 (Group IV)



Supplementary Figure 2: FISH with combination of 5S rDNA, 35S rDNA, and satellite DNA *PaB6* in allotetraploids of B⁶ and B⁷ origin in *Prospero autumnale* complex allows identification of parental origin of chromosomes carrying 5S and 35S rDNA in most allopolyploids (Groups II-IV): (A) group I; $2n = 25$: 35S rDNA (green) and *PaB6* satellite (red) spread over all chromosomes, with 22 strong and 3 weak signals (H208); (B-F) $2n = 28$. (B) Group II, 35S rDNA (green) in two chromosomes 3 contributed by maternal B⁷ genome (lacking amplified *PaB6* signals; red) (H434). (C) Group II, two 35S rDNA signals (green) in two chromosomes 3 contributed by B⁷ paternal genome lacking *PaB6* signals and one weak 35S rDNA (arrow in inset) in one of two maternal chromosomes 3 exhibiting strong *PaB6* signals (H363). (D-E) Group III, chromosomes of the same cell after reprobing (H238): (D) 5S rDNA (green) and 35S rDNA (red), arrow indicated very weak signal, (E) 5S rDNA (green) and *PaB6* (red). Parental origin of all rDNA bearing chromosomes can be inferred (see also Table 1). (F) Group IV, three 35S rDNA signals (green) in three chromosomes 3 contributed by maternal genome B⁷ and one weak 35S rDNA signal (arrow) in chromosome 3 contributed by paternal parent (Table 1), *PaB6* in red (H152). (G-H): individual H178 $2n = 26$ with 18 spread signals: (G) two 35S rDNA signal (green) in chromosomes with strong *PaB6* signals (red; of B⁶ genome origin), and two 35S rDNA signals in two chromosomes 3 lacking *PaB6* signals contributed by B⁷ genome. (H) four 5S¹ rDNA loci in two chromosomes with *PaB6* (red) and two lacking *PaB6*, four 5S² rDNA loci (all in chromosomes with *PaB6*), and 18 strong *PaB6* signals (H178). Arrows indicate weak signals of 35S rDNA. Scale bar, 5 μ m.



Supplementary Figure S3. Variable (parsimony informative) nucleotide positions in ITS DNA sequence alignment.

AA/AAB⁷B⁷/B⁷B⁷

P. autumnale	H541	AA	C	T	T	T	T	A	C	T	T	C	C	A	A	C	T	C
P. autumnale	H543	AA	C	T	T	T	T	A	C	T	T	C	C	A	A	C	T	C
P. autumnale	H550	AA	C	T	T	T	T	A	C	T	T	C	C	A	A	C	T	C
P. autumnale	H557	AA	C	T	T	T	T	A	C	T	T	C	C	A	A	C	T	C
P. autumnale	H603	AAB ⁷ B ⁷	T	C	C	C	G	G	T	---	---	T	C	G	A	A	G	---
P. autumnale	H607	AAB ⁷ B ⁷	T	C	C	C	G	G	T	---	---	T	C	G	A	A	G	---
P. autumnale	H447	B ⁷ B ⁷	T	C	A	C	G	G	T	---	---	T	C	G	A	A	G	---
P. autumnale	H575	B ⁷ B ⁷	T	C	C	C	G	G	T	---	---	T	C	G	A	A	G	---
P. autumnale	H576	B ⁷ B ⁷	T	C	C	C	G	G	T	---	---	T	C	G	A	A	G	---
P. autumnale	H239	B ⁷ B ⁷	T	C	C	C	G	G	T	---	---	T	C	G	A	A	G	---
P. autumnale	H428	B ⁷ B ⁷	T	C	A	C	G	G	T	---	---	T	C	G	A	A	G	---
P. autumnale	H612	B ⁷ B ⁷	T	C	C	C	G	G	T	---	---	T	C	G	A	A	G	---

The following positions in alignment are shown: 108, 194, 295, 320, 331, 333, 458, 551, 559, 560, 575, 621, 641, 557, 665, 731.

B⁷B⁷/B⁷B⁷B⁷B⁷

P. autumnale	H132	B ⁷ B ⁷ B ⁷ B ⁷	C	T
P. autumnale	H534	B ⁷ B ⁷ B ⁷ B ⁷	C	T
P. autumnale	H172	B ⁷ B ⁷ B ⁷ B ⁷	C	G
P. autumnale	H230	B ⁷ B ⁷ B ⁷ B ⁷	C	G
P. autumnale	H310	B ⁷ B ⁷ B ⁷ B ⁷	C	G
P. autumnale	H401	B ⁷ B ⁷ B ⁷ B ⁷	C	G
P. autumnale	H435	B ⁷ B ⁷ B ⁷ B ⁷	A	G
P. autumnale	H577	B ⁷ B ⁷ B ⁷ B ⁷	C	G
P. autumnale	H615	B ⁷ B ⁷ B ⁷ B ⁷	C	G
P. autumnale	H628	B ⁷ B ⁷ B ⁷ B ⁷	C	G
P. autumnale	H447	B ⁷ B ⁷	A	G
P. autumnale	H575	B ⁷ B ⁷	C	T
P. autumnale	H576	B ⁷ B ⁷	C	G
P. autumnale	H239	B ⁷ B ⁷	C	G
P. autumnale	H428	B ⁷ B ⁷	A	G
P. autumnale	H612	B ⁷ B ⁷	C	G

The following positions in alignment are shown: 395, 311.

B⁷B⁷ /B⁶B⁶/B⁶B⁶B⁷B⁷

P. autumnale	H239	B7B7	TGTCGCTGCGAG
P. autumnale	H428	B7B7	TGTCGCTGCGAG
P. autumnale	H447	B7B7	TGTCGCTGCGAG
P. autumnale	H575	B7B7	TGTCGCTGCGAG
P. autumnale	H576	B7B7	TGTCGCTGCGAG
P. autumnale	H612	B7B7	TGTCGCTGCGAG
P. autumnale	H152	B6B6B7B7	TGTCGCTGCGAG
P. autumnale	H355	B6B6B7B7	TGTCGCTGCGAG
P. autumnale	H356	B6B6B7B7	TGTCGCTGCGAG
P. autumnale	H388	B6B6B7B7	TGTCGCTGCGAG
P. autumnale	H410	B6B6B7B7	TGTCGCTGCGAG
P. autumnale	H434	B6B6B7B7	TGTCGCTGCGAG
P. autumnale	H238	B6B6B7B7 clone1	TGTCGCTGCGAG
P. autumnale	H166	B6B6	CTCCTACATATC
P. autumnale	H170	B6B6	CTCCTACATATC
P. autumnale	H195	B6B6	CTCCTACATATC
P. autumnale	H274	B6B6	CTCCTACATATC
P. autumnale	H408	B6B6	CTCCTACATATC
P. autumnale	H427	B6B6	CTCCTACATATC
P. autumnale	H468	B6B6	CTCCTACATATC
P. autumnale	H14	B6B6B7B7	CTCCTACATATC
P. autumnale	H96	B6B6B7B7	CTCCTACATATC
P. autumnale	H153	B6B6B7B7	CTCCTACATATC
P. autumnale	H178	B6B6B7B7	CTCCTACATATC
P. autumnale	H207	B6B6B7B7	CTCTTCCATATC
P. autumnale	H208	B6B6B7B7	CTCCTACATATC
P. autumnale	H238	B6B6B7B7 clone2	CTCTTCCATATC
P. autumnale	H300	B6B6B7B7	CTCTTCCATATC
P. autumnale	H363	B6B6B7B7	CTCCTACATATC
P. autumnale	H238	B6B6B7B7	YKYYKCYRYRWS
P. autumnale	H331	B6B6B7B7	YKYYKCYRYRWS

The following positions in alignment are shown: 108; 187; 228; 316; 331; 360; 575; 610; 632; 641; 665; 736.

Supplementary Table 1: Plant material of *Prospero* studied with collection details, chromosomes numbers, genome size and GenBank accession numbers (ITS and plastid DNA).

Cytotype	Locality; Collection; accession number	2n	GenBank accession numbers
			ITS / Plastid
Outgroups			
<i>Prospero obtusifolium</i>	Spain; Parker; H540	8 ¹	KC899275
	Spain; Parker; H559	8 ¹	KC899272
	Morocco; Parker; H547	8 ¹	KC899273
<i>P. hanburyi</i>	Turkey, Findikpinar; Speta; H115	14 ¹	KC899269
	Turkey, Findikpinar; Speta; H397	14 ¹	KC899271
	Turkey, Narlikuyu; Silifke; H231	14 ¹	KC899270
<i>P. autumnale</i> complex			
Diploids			
AA	Spain, Huelva; Parker; H541	14 ¹	KC899278
	Spain, Badajoz; Parker; H543	14 ¹	KC899279
	Spain, Huelva; Parker; H557	14 ¹	KC899282
	Portugal, Peniche; Parker; H550	14 ¹	KC899281
B ⁷ B ⁷ (single 5SrDNA)	Greece, Naxos; Speta; H575	14 ¹	KC899300
	Israel, Nene Han; Parker; H612	14 ¹	KC899301
	Italy, Sicily; Speta; H428	14 ¹	KC899298
	Speta; H447	14 ¹	KC899299
	Cyprus; Speta; H239	14 ¹	KC899297
B ⁷ B ⁷ (duplicated 5S rDNA)	Serbia, Siget-Baun; Rat; H576	14 ¹	KC899303

Supplementary Table 1. continued

B ⁶ B ⁶	Greece, Crete; Speta; H166	12 ¹	KC899284
	Greece, Crete; Speta; H170	12 ¹	KC899285
	Greece, Crete; Speta; H195	12 ¹	KC899290
	Greece, Crete; Speta; H274	12 ¹	KC899291
	Greece, Crete; Jahn; H408	12 ¹	KC899288
	Greece, Crete; Jahn & Böhling; H427	12 ¹	KC899293
	Greece, Crete; Speta; H468	12 ¹	KC899292
B ⁵ B ⁵	Libya, Mt. Tobi; Parker; H566	10 ¹	KC899313/
	Libya, Mt. Tobi; Parker; H581	10 ¹	KC899314/
	Libya, Mt. Tobi; Parker; H582	10 ¹	KC899316
	Libya, Mt. Tobi; Parker; H637	10 ¹	KC899312
	Libya, Nagasa; Parker; H640	10 ¹	KC899315/
Polyploids			
AAB ⁷ B ⁷	Portugal, Algarve; Parker; H603	28	
	Portugal, Algarve; Parker; H607	28	
B ⁶ B ⁶ B ⁷ B ⁷	Greece, Crete; Speta; H153	25	
	Greece, Crete; Weigl; H208	25	
	Greece, Crete; Speta; H14	26	
	Greece, Crete; Speta; H96	26	
	Greece, Crete; Speta; H207	27	
	Greece, Crete; Speta; H152	28	
	Greece, Crete; Speta; H238	28	
	Greece, Crete; Speta; H300	28	

	Greece, Crete; Raus; H331	28
Supplementary Table 1. continued		
	Greece, Crete; Speta; H355	28
	Greece, Crete; Passauer; H356	28
	Greece, Crete; Böhling; H363	28
	Greece, Crete; Raus; H388	28
	Greece, Crete; Jahn; H410	28
	Greece, Crete; Speta; H434	28
B ⁷ B ⁷ B ⁷ B ⁷	Croatia, Kornati; Parker; H628	28
	France, Morbihan; Ragot; H615	28
	Greece, Euboea; Speta; H230	28
	Greece, Kos; Speta; H310	28
	Greece, Zakynthos; Speta; H132	28
	Italy, Sicily; Speta; H435	28
	Italy, Sardinia; Grims; H534	28
	Montenegro, Kotor; Rat; H577	28
	Republic of Malta; Speta; H401	28
	Spain, Minorca; Speta; H172	28

*: measured only one time due to limited amount of material; -: not measured.

**B-chromosomes in *Prospero autumnale* complex (Hyacinthaceae):
structural polymorphisms and distinct repeat composition suggest
their recurrent origin and ongoing evolution**

Tae-Soo Jang¹, John Parker², Hanna Weiss-Schneeweiss¹

¹Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14,
A-1030 Vienna, Austria;

²Cambridge University Botanic Garden, Cambridge, CB2 1JF, UK

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ABSTRACT

- *Background and Aims* Supernumerary B-chromosomes (Bs) are parasitic established components of some genomes, which do not recombine with the regular A-complement chromosomes and follow their own evolutionary trajectories. B-chromosomes originate from regular A-complement following chromosomal rearrangements. They are composed of various parts of the A-chromosomes, and may contain significant amounts of organellar and repetitive DNAs acquired from various parts of the host genome or B-specific repeats which have evolved *de novo*.
- *Methods* The genomic composition, origin and evolution of B-chromosomes can be assessed in the chromosomally variable *Prospero autumnale* complex. The composition of B-chromosomes from twenty-two individuals, representing three of the four basic diploid cytotypes and their auto- and allopolyploid derivatives, have been analyzed using fluorescence *in situ* hybridization (FISH) with three types of tandem repeats, two rDNAs and a satellite DNA *PaB6*.
- *Key Results* High levels of variation in copy number and distribution of the three repeats were found in Bs, within and between cytotypes and even within individuals. B-chromosomes in diploids usually lacked 35S rDNA except in one individual whose Bs were enriched in 35S rDNA but possessed no 5S rDNA. Bs occurring in most diploids and all polyploids, by contrast, were typically enriched in 5S rDNA. The satellite DNA *PaB6* localizes to pericentric regions of standard chromosomes with copy numbers being cytotype-specific and maps to pericentromeric and subtelomeric regions of nearly all B-chromosomes. The quantity of *PaB6* in Bs does not correlate with that in the standard chromosomes, but positively correlates with ploidy level. A combination of *PaB6* and 5S rDNA painted whole B-chromosomes in higher polyploids.

- *Conclusions* Eleven combinations of rDNA and *PaB6* distribution were observed in Bs in only 22 individuals of *P. autumnale*. This remarkable variation, interpreted in the context of the extraordinarily high levels of chromosomal variation in *P. autumnale* suggests independent and recurrent origins of Bs, as by-products of extensive genome restructuring.

Key words: B-chromosome evolution, FISH (fluorescence *in situ* hybridization), *Prospero autumnale* complex, rDNA (5S and 35S rDNA), satellite DNA *PaB6*.

INTRODUCTION

Karyotypes of many species contain supernumerary genetic materials. It can be present as free B-chromosomes (Bs) or can be inserted on standard chromosomes as supernumerary chromosomal segments (SCS). SCSs can be identified in the heterozygous condition due to homologous chromosomes length differences (John and Miklos, 1979; Weiss-Schneeweiss and Schneeweiss, 2013). They are frequent in insects, but are also found in a few plants, most notably in the monocotyledonous family Hyacinthaceae (Greilhuber and Speta, 1978; Ruiz Rejón and Oliver, 1981; Jamilena *et al.*, 1995; Ebert *et al.*, 1996; Garrido-Ramos *et al.*, 1998; Weiss-Schneeweiss *et al.*, 2004).

By contrast, B-chromosomes have been reported in numerous species of animals and fungi (Camacho *et al.*, 2000) and about 10–15% of flowering plant species (Jones, 1995). They 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 more common in monocots (8%) than in dicots (3%), with hot spots in Liliales and Commelinales (Levin *et al.*, 2005). B frequencies in diploids and polyploids are similar (Jones and Rees, 1982; Trivers *et al.*, 2004), but it has been suggested that they may be higher in families with larger genome sizes (Trivers *et al.*, 2004; Levin *et al.*, 2005; Jones *et al.*, 2008).

B-chromosomes are most frequently found in low numbers (0–5), but as many as 34 Bs have been reported in an individual of *Zea mays* (Jones and Rees, 1982; Jones *et al.*, 2008). They are usually smaller than the standard complement and vary in size from dot-like micro Bs (Carter and Smith-White, 1972; Houben *et al.*, 1997; Ding *et al.*, 1998; see Jones *et al.*, 2008; Houben *et al.*, 2013) to chromosomes as large as the smallest chromosomes of the regular set (Carter and Smith-White, 1972; Jones *et al.*, 2008). Their size and structure is usually stable within taxa (*Secale cereale*: Jones and Puertas, 1993; but see Marques *et al.*,

2012), but individuals carrying more than one structural type of Bs are also known (Guillén and Ruiz Rejón, 1984; Parker *et al.*, 1991).

The occurrence of B-chromosomes 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; Jones *et al.*, 2008; Martis *et al.*, 2012; Houben *et al.*, 2013; Weiss-Schneeweiss and Schneeweiss, 2013), with the most favoured invoking their origin as a byproduct of chromosomal rearrangements of A-chromosomes stimulated by hybridization or polyploidization (Jones and Houben, 2003; Houben *et al.*, 2013). Support for this hypothesis has come from the genera *Plantago* (Dhar *et al.*, 2002) and *Secale* (Martis *et al.*, 2012). Newly-arisen chromosomal fragments must accumulate sufficient differences in structure and chromatin composition to ensure their meiotic isolation from A-chromosomes (Langdon *et al.*, 2000; Marschner *et al.*, 2007). Evolutionarily successful B-chromosomes must secure their own transmission to the next host generations and this is achieved by mechanisms of mitotic and meiotic drive (Jones and Houben, 2003; Jones *et al.*, 2008).

During their evolution, Bs capture A-chromosome derived coding and non-coding DNA (Małuszyńska and Schweizer, 1989; Dhar *et al.*, 2002; Kubaláková *et al.*, 2003; Camacho, 2005; Carchilan *et al.*, 2009; Peng and Cheng, 2011; Banaei-Moghaddam *et al.*, 2012; Marques *et al.*, 2012) and organellar DNA sequences (Martis *et al.*, 2012), but novel types of B-specific repeats also evolve (Langdon *et al.*, 2000; Martis *et al.*, 2012). Despite their abundance, the roles of Bs remains elusive, although various group-specific 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 B-chromosome evolution is in the genus *Prospero* (Hyacinthaceae). The *P. autumnale* complex, one of the three species of this genus includes four evolutionarily well-established diploid cytotypes (AA, B⁷B⁷, B⁵B⁵, B⁶B⁶;

Jang *et al.*, 2013). Each cytotype is characterized by a unique combination of basic chromosome numbers, genome size, and patterns of rDNA and satellite DNA *PaB6* distribution (Jang *et al.*, 2013 and Chapter 2). Polyploidy is frequent in the complex resulting in autopolyploids of genome B⁷ (most commonly 4x and 6x, but up to 20x; Ainsworth, 1983; Ebert, 1993; Speta, 1993, 2000) and two classes of allopolyploids - of A and B⁷ origin or of B⁶ and B⁷ origin (Jang *et al.*, Chapter 3). B-chromosomes have been reported from three of the four diploid cytotypes with the exception of the youngest cytotype B⁵B⁵ (Ruiz Rejón *et al.*, 1980; Ebert *et al.*, 1996; Taylor, 1997), and in a range of polyploids (Ebert, 1993; Taylor, 1997). Bs of *Prospero* vary in size and structure, even within single cells (Ruiz Rejón *et al.*, 1980; Parker *et al.*, 1991; Ebert, 1993; Taylor, 1997).

Recent studies of the *P. autumnale* complex have provided a phylogenetic framework and insights into the evolutionary dynamics of diploids and polyploids (Jang *et al.*, 2013 - Chapters 1, 2, 3). They have also provided tools with which to analyze the evolution of B-chromosomes and SCSs.

One aim of this study is to analyze B-chromosomes structure and composition in individuals of different diploid and polyploid cytotypes of the chromosomally remarkably variable *P. autumnale* complex. Two tandem repeats derived from 35S and 5S rDNA and a genus-specific satellite DNA *PaB6* have been used. *PaB6* is a dynamic component of *Prospero* genomes which accompanies diploid and polyploid evolution. Correlations between the degree of amplification of *PaB6* in the standard complement and B-chromosomes will also be presented.

MATERIALS AND METHODS

Plant material

Twenty two plants of the *Prospero autumnale* complex containing B-chromosomes (Bs) were analyzed (Table 1). 11 individuals were diploid, three of cytotype AA, six of B⁷B⁷, one of B⁶B⁶, and one diploid homoploid hybrid B⁶B⁷, and 11 polyploid, three allopolyploids of B⁶ and B⁷ origin and eight autopolyploids of genome B⁷. For cytological investigations, root meristems were pretreated with a solution with 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 : acetic acid : chloroform (6 : 3 : 1) and stored at –20°C.

Karyotyping and fluorescence in situ hybridization (FISH)

Chromosome numbers and karyotypes were analyzed as described by Jang *et al.* (2013; Chapter 1) using standard Feulgen staining. Chromosomal spreads for FISH were prepared by enzymatic digestion and squashing as described in Jang *et al.* (2013 - Chapter 1, Chapter 3). Flower buds of plants with B⁷B⁷ + 5Bs and B⁷B⁷ + 2Bs were digested with 1% cellulase Onozuka (Serva, Heidelberg, Germany), 1% cytohelicase (Sigma-Aldrich, Vienna, Austria), and 1% pectolyase (Sigma-Aldrich) for 70 min at 37°C.

Probes used for FISH were: satellite DNA *PaB6* isolated from the B⁶ genome in plasmid pGEM-T easy (Jang *et al.*, Chapter 2), 35S rDNA (18S/25S rDNA) from *Arabidopsis thaliana* in plasmid pSK+, and 5S rDNA from *Melampodium montanum* in plasmid pGEM-T easy, directly labeled with biotin or digoxigenin (Roche, Vienna, Austria). Probes were labeled either directly by PCR (5S rDNA and satellite DNA *PaB6*) or using a nick translation kit (35S rDNA; Roche, Vienna, Austria). Digoxigenin was detected with antidigoxigenin conjugated with FITC (5 µg mL⁻¹; Roche, Vienna, Austria) and biotin with ExtrAvidin conjugated with Cy3 (2 µg mL⁻¹; Sigma-Aldrich, Vienna, Austria). Preparations were analyzed with an AxioImager M2 epifluorescent microscope (Carl Zeiss, Vienna, Austria),

images captured with a CCD camera and processed using AxioVision ver. 4.8 (Carl Zeiss, Vienna, Austria) with only those functions that apply to all pixels of the image equally.

TABLE 1. Plant material 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
AA+3Bs	Spain, Jaén; Parker; H546	17
B ⁷ B ⁷ +1B	Greece, Crete; Speta; H209	15
B ⁷ B ⁷ +2Bs	Greece, Crete; Speta; H526	16
	Montenegro; Speta; H415*	16
B ⁷ B ⁷ +4Bs	Greece, Skopelos; Parker; H620	18
B ⁷ B ⁷ +5Bs	Montenegro; Speta; H412*	19
B ⁷ B ⁷ +6Bs	Montenegro; Speta; H413	20
B ⁶ B ⁶ +1B	Greece, Crete; Speta; H154-1	13
B ⁶ B ⁷ +2Bs	Greece, Crete; Weigl; H246	15
Polyploids		
B ⁶ B ⁶ B ⁷ B ⁷ + 1B	Greece, Crete; Speta; H213	28
B ⁶ B ⁶ B ⁷ B ⁷ + 2Bs	Greece, Crete; Raus; H327	30
B ⁷ B ⁷ B ⁷ B ⁷ + 1B	Montenegro; Speta; H384	29
	Spain, Biscay; Parker; H624	29
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 1B	Greece, Crete; Jahn; H339-1	36
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 3Bs	Greece, Karpachos; Raus; H336	38
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 4Bs	Greece, Crete; Jahn; H159	39
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 1B	Greece, Crete; Speta; H536	43
B ⁶ B ⁶ B ⁷ B ⁷ B ⁷ B ⁷ + 3Bs	Greece, Crete; Speta; H121	45
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 4Bs	Tunisia; Speta; H303	46
	Tunisia; Speta; H405	46

* used for meiotic analyses

RESULTS

The number of Bs varied from one to six per individual, with one B-chromosome most frequent. All Bs were acro-, meta, and submetacentric (Supplementary Data Fig. S1) and their size varied from 1.81 μm to 4.79 μm (Table 2). The size and shape of Bs was uniform in AA individuals, but in B^7B^7 diploids varied in size and morphology (Table 2). Bs in B^7 autopolyploids were most variable, and tended to be larger than in those in diploids (Table 2).

TABLE 2. The size of B chromosomes in *Prospero autumnale* complex. Bs were measured from Feulgen stained preparations (Jang *et al.*, 2013). B chromosome morphology: ^aacrocentric, ^mmetacentric, and ^ssubmetacentric.

Cytotype	2n	Absolute length of B chromosomes (μm)						Type of Bs	Figures	Accession number
		1	2	3	4	5	6			
<i>P. autumnale</i> complex										
Diploids										
AA + 1B	14+1B	2.78 ^m						1a	1A, 2A	H549
AA + 2Bs	14+2Bs	2.05 ^m	2.14 ^m					1a	1B, 2B	H560
AA + 3Bs	14+3Bs	2.49 ^s	2.74 ^s	2.76 ^s				1b	1C, 2C	H546
B ⁶ B ⁶ + 1B	12+1B	2.92 ^s						4	1I, 2J	H154-1
B ⁶ B ⁷ + 2Bs	13+2Bs	2.55 ^s	2.61 ^s					6, 5	1J, 2K	H246
B ⁷ B ⁷ + 1B	14+1B	3.37 ^s						2	1D, 2D	H209
B ⁷ B ⁷ + 2Bs	14+2Bs	2.14 ^s	2.20 ^s					7	4B	H415
B ⁷ B ⁷ + 2Bs	14+2Bs	2.80 ^m	2.93 ^m					7	1E, 2E	H526
B ⁷ B ⁷ + 4Bs	14+4Bs	2.71 ^a	2.90 ^a	2.94 ^m	3.20 ^s			7	1F, 2F	H620
B ⁷ B ⁷ + 5Bs	14+5Bs	1.81 ^s	1.88 ^a	1.88 ^s	1.92 ^s	2.28 ^s		3	1G, 2G-H, 4A	H412
B ⁷ B ⁷ + 6Bs	14+6Bs	2.07 ^a	2.11 ^s	2.21 ^m	2.22 ^a	2.32 ^m	2.34 ^s	7	1H, 2I	H413
Polyploids										
B ⁶ B ⁶ B ⁷ B ⁷ + 1B	27+1B	2.63 ^m						8	1P, 2S	H213
B ⁶ B ⁶ B ⁷ B ⁷ + 2Bs	28+2Bs	1.82 ^s	2.11 ^s					7	2T	H327
B ⁷ B ⁷ B ⁷ B ⁷ + 1B	28+1B	1.88 ^m						9	1L, 2M	H384
B ⁷ B ⁷ B ⁷ B ⁷ + 1B	28+1B	4.79 ^s						4	1K, 2L	H624
B ⁷ B ⁷ B ⁷ B ⁷ + 1B	35+1B	2.02 ^m						10	1M, 2N	H339-1
B ⁷ B ⁷ B ⁷ B ⁷ + 3Bs	35+3Bs	1.75 ^s	2.47 ^m	2.78 ^s				8	2O	H336
B ⁷ B ⁷ B ⁷ B ⁷ + 4Bs	35+4Bs	3.00 ^m	3.05 ^s	3.21 ^s	3.27 ^s			7	1N	H159
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 1B	42+1B	3.29 ^m						11	1Q, 2P	H536
B ⁶ B ⁶ B ⁷ B ⁷ B ⁷ + 3Bs	42+3Bs	-	-	-				5	2U	H121
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 4Bs	42+4Bs	-	-	-	-			7	2Q	H303
B ⁷ B ⁷ B ⁷ B ⁷ B ⁷ + 4Bs	42+4Bs	2.43 ^m	2.54 ^m	2.91 ^m	3.10 ^m			7	2R	H405

Composition of B-chromosomes: repetitive DNA

35S rDNA, 5S rDNA, and satellite DNA *PaB6* tandem repeats were mapped in mitotic and meiotic chromosomes of the standard complement and in Bs (Figs. 1–3 and Tables 1–2).

Plastid-derived sequences gave no signals.

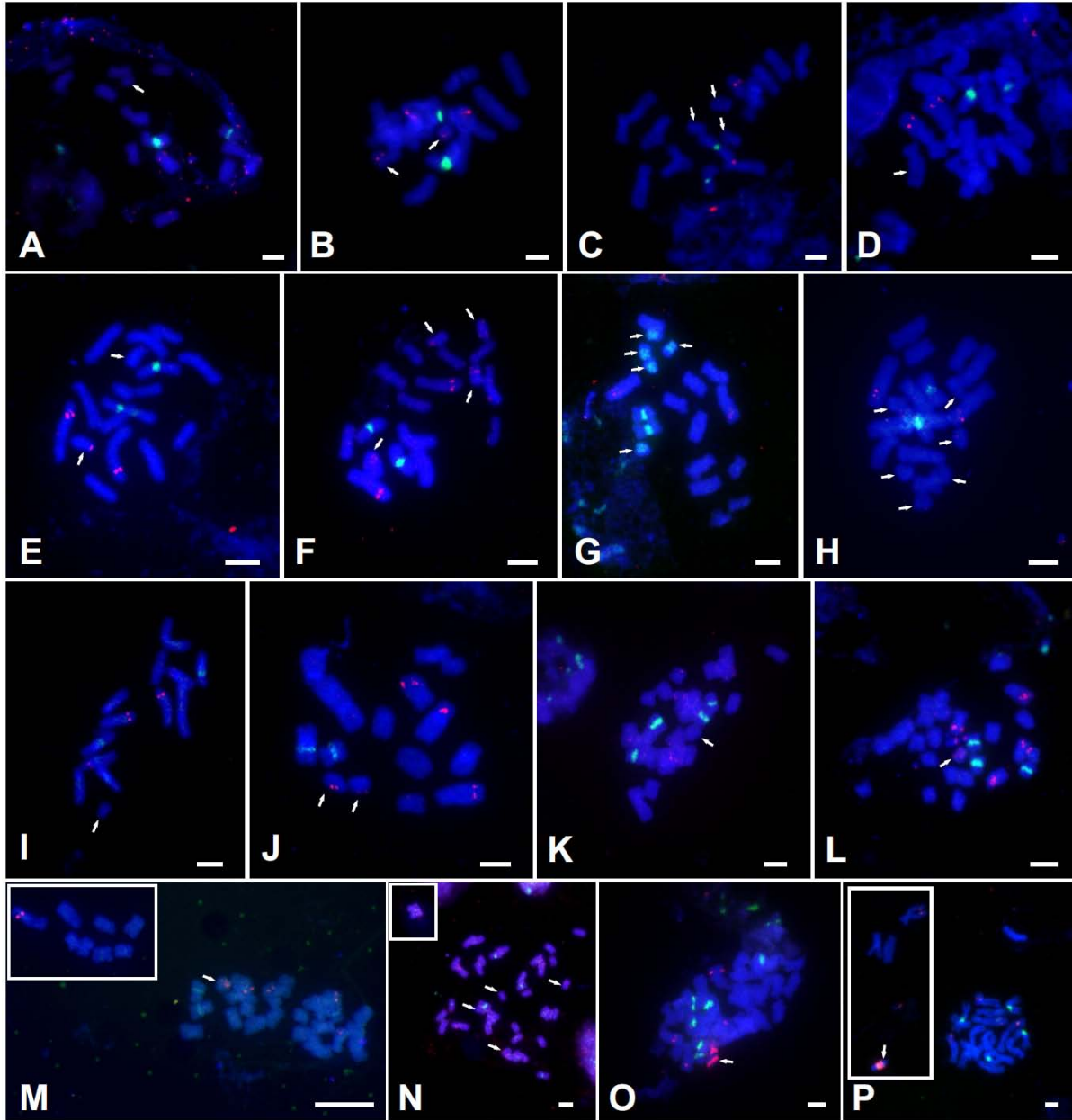


FIG. 1. Localization of 35S (green) and 5S rDNA loci (red) in B-chromosomes of diploid (A-J) and polyploid (K-P) individuals of *Prosopero autumnale* complex. (A) AA + 1B (H549). (B) AA + 2Bs (H560). (C) AA + 3Bs (H546). (D) B⁷B⁷ + 1B (H209). (E) B⁷B⁷ + 2Bs (H526). (F) B⁷B⁷ + 4Bs (H620). (G) B⁷B⁷ + 5Bs (H412). (H) B⁷B⁷ + 6Bs (H413). (I) B⁶B⁶ + 1B (H154-1). (J) B⁶B⁶ + 2Bs (H246). (K) B⁷B⁷B⁷B⁷ + 1B (H624). (L) B⁷B⁷B⁷B⁷ + 1B (H384). (M) B⁷B⁷B⁷B⁷ + 1B (H339-1). (N) B⁷B⁷B⁷B⁷ + 4Bs (H159). (O) B⁷B⁷B⁷B⁷B⁷ + 1B (H536). (P) B⁶B⁶B⁷B⁷ + 1B (H213). Insets in (M), (N), and (P) show chromosomes of a single cell which were lying at some distance from the main chromosome group and either could not be photographed together using high magnification objectives or were too far apart to clearly demonstrate chromosome morphology while showing the whole field. Arrows indicate Bs. Scale bar = 5 μ m.

35S and 5S rDNA repeats

Only one plant had B-chromosomes with 35S rDNA. All five B-chromosomes in this individual showed signals spread over the whole short arm and part of the long arm (Figs. 1G and 3 Type 3). No secondary constrictions, however, were detected.

5S rDNA, by contrast, was detected in the majority of B-chromosomes with the exception of Bs in two B^7B^7 diploids (Fig. 3). The 5S rDNA signals appeared weak and dot-like (Figs. 1A–J and 3 Types 1–7). Bs in polyploid individuals were also enriched in 5S rDNA repeats (Figs. 1O–P and 3 Types 8, 11), with the copy number increasing with ploidy level (Fig. 3). In the extreme case, the 5S rDNA probe painted more than half the B-chromosome, mainly in the centromeric and pericentromeric regions (H213, Figs. 1P and 3 Type 8). Interestingly, the five Bs with 35S rDNA had no 5S rDNA amplification.

Satellite DNA PaB6

The satellite DNA *PaB6* was found in B-chromosomes of diploids and polyploids, although enhanced and more variable in polyploids. This ploidy level-correlated amplification/accumulation (Fig. 2 and Table 2) was particularly evident in autopolyploids of genome B^7 where entire Bs were painted by *PaB6* (Figs. 2–3). *PaB6* and 5S rDNA were often both amplified in Bs, but usually preferentially occupied separate chromosomal regions (Fig. 3).

In B-chromosomes of diploids, copy number and distribution of *PaB6* was variable. Thus B-chromosomes in cytotype AA were uniform both in structure and in *PaB6* distribution (Fig. 3), where signals were consistently present in subterminal regions of both chromosomal arms (Figs. 2A–C and 3 Type 1a–b) although strengths could differ (Fig. 3). The B-chromosome of the single B^6B^6 plant showed very little signal with *PaB6* (Figs. 2J and 3 Type 4).

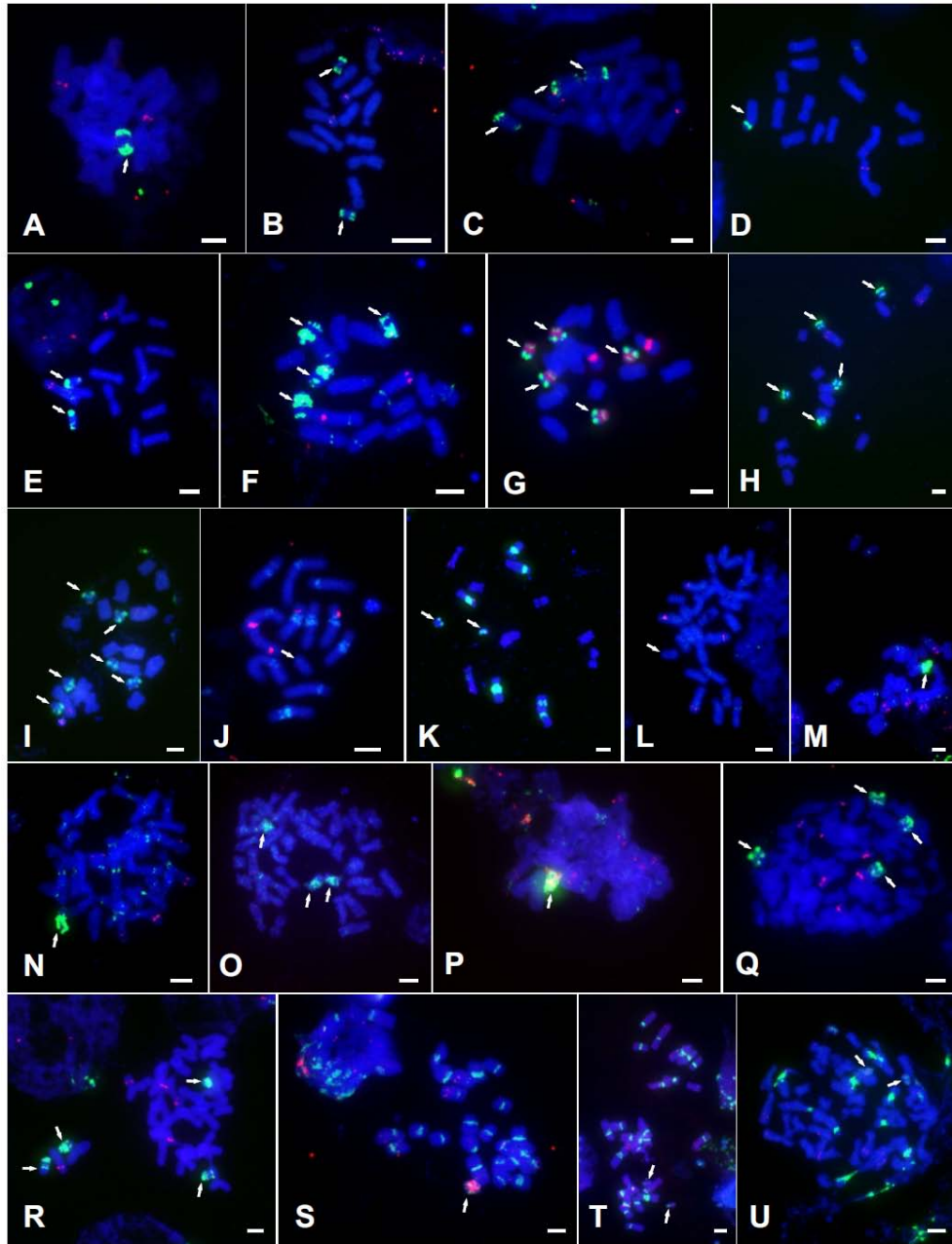


FIG. 2. Localization of 5S rDNA (red signals) and satellite DNA *PaB6* loci (green signals) in B-chromosomes of diploid (A–K) and polyploid (L–U) individuals *Prospero autumnale* complex. Metaphase chromosomes were subjected to FISH with 5S rDNA (red in all except for G where red depicts 35S rDNA) and *PaB6* (green). (A) AA + 1B (H549). (B) AA + 2Bs (H560). (C) AA + 3Bs (H546). (D) B⁷B⁷ + 1B (H209). (E) B⁷B⁷ + 2Bs (H526). (F) B⁷B⁷ + 4Bs (H620). (G–H) B⁷B⁷ + 5Bs (H412). (I) B⁷B⁷ + 6Bs (H413). (J) B⁶B⁶ + 1B (H154–1). (K) B⁶B⁷ + 2Bs (H246). (L) B⁷B⁷B⁷B⁷ + 1B (H624). (M) B⁷B⁷B⁷B⁷ + 1B (H384). (N) B⁷B⁷B⁷B⁷B⁷ + 1B (H339–1). (O) B⁷B⁷B⁷B⁷B⁷ + 3Bs (H336). (P) B⁷B⁷B⁷B⁷B⁷B⁷ + 1B (H536). (Q) B⁷B⁷B⁷B⁷B⁷B⁷ + 4Bs (H303). (R) B⁷B⁷B⁷B⁷B⁷B⁷ + 4Bs (H405). (S) B⁶B⁶B⁷B⁷ + 1B (H213). (T) B⁶B⁶B⁷B⁷ + 2Bs (H327). (U) B⁶B⁶B⁷B⁷B⁷B⁷ + 3Bs (H121). Scale Bar = 5 μ m. Arrows indicate Bs.

Bs in diploid and polyploid B^7B^7 individuals showed great variation in *PaB6* signals (Figs. 2D–I and 3 Types 2–3, 6–7). One B-chromosome variant with a strong subterminal signal in the long arm, a weaker short arm signal and occasional pericentric signal was present in three diploids and three autopolyploids (Figs. 2E–I and 3 Type 3, 6–7). A single diploid individual carried a putative B-chromosome with clear pericentric localization of *PaB6* typical of the standard complement chromosomes (Figs. 2D and 3 Type 2) and lacked the *PaB6* speckles often observed while a B^6B^7 hybrid carried two different B-chromosomes (H246, Figs. 2K, and 3 Type 5–6). The Bs of B^7 autopolyploids were extremely variable with signals ranging from near-absence (Fig. 2L) to high levels of accumulation (Fig. 2M).

Meiotic analyses

Meiotic pairing of B-chromosomes in two diploid B^7B^7 individuals revealed different patterns of pairing (Fig. 4). The five Bs present in H412 were univalent. They carried 35S rDNA and satellite DNA *PaB6* (Fig. 4A). By contrast, the two B-chromosomes of H415 had amplified *PaB6* and 5S rDNA and formed a bivalent (Fig. 4B).

DISCUSSION

Structural variation of B-chromosomes in the Prospero autumnale complex

The B-chromosomes of the *Prospero autumnale* complex are highly variable in structure and repeat composition. Despite the small number analyzed, the B-chromosomes could be assigned to 11 types based on structure and, mainly, the distribution of rDNA and *PaB6* repeats (Fig. 3).

As is usual in plants, the B-chromosomes of *Prospero* are smaller than the smallest chromosomes of the standard chromosome complement (Johnson, 2003; Weiss-Schneeweiss *et al.*, 2009; Alves *et al.*, 2011; Gao *et al.*, 2011), although one putative B-chromosome fell in

the range of the standard chromosomes (Fig. 1D and Table 2; H624). It is not clear, however, whether this chromosome represents an established B-chromosome.

The structural variation of Bs in the current study represents only a subset of that already described (Ruiz Rejón *et al.*, 1980; Parker *et al.*, 1991; Ebert, 1993; Taylor, 1997). Telocentrics, acrocentrics, and metacentrics have been documented (Ruiz Rejón *et al.*, 1980; Hong, 1982; Guillén and Ruiz Rejón, 1984; Parker *et al.*, 1991; Ebert *et al.*, 1996). Population studies of B-chromosomes in *P. autumnale* have been initiated to address the relationship of their variation and ploidy level of the host plant directly.

The B-chromosomes in the AA cytotype in this study were all similar in structure and in *PaB6* and 5S rDNA content (Fig. 3 and Table 2). A structurally similar type was reported earlier in AA and was the dominant variant (Parker *et al.*, 1991). Cytotype B⁷B⁷ shows the highest level of structural B-variation (Ebert, 1993; Ebert *et al.*, 1996), perhaps reflecting their origins in different geographic or evolutionary lineages. B⁷B⁷ is the only widespread cytotype while all other have more or less restricted distributions (Vaughan *et al.*, 1997; Jang *et al.*, 2013). The DNA repeat studies reflect the structural variation. Previous studies have reported submetacentric GC-rich Bs with pericentric C-bands, and acrocentric Bs with heterochromatin present either as blocks or speckles in subterminal, interstitial and pericentromeric regions (Ebert, 1993). B-chromosomes have previously been reported in B⁷ autopolyploids of *Prospero* (Ebert, 1993; Taylor, 1997; Ainsworth, pers. comm.) and in AAB⁷B⁷ allotetraploids (Parker *et al.*, 1991).

	A				B				Cytotype (accesion number)
	DAPI	5S	35S	Merged	DAPI	5S	PaB6	Merged	
Type 1a									 AA (H560)*
Type 1b									 AA (H546)*
Type 2									 B'B' (H209)*
Type 3									 B'B' (H412)*
Type 4									 B'B' (H154-1)* B'B'B' (H624)
Type 5									 B'B' (H246)* B'B'B'B'B' (H121)
Type 6									 B'B' (H246)*
Type 7									 B'B' (H413, H526*, H620), B'B'B' (H327), B'B'B'B'B' (H159), B'B'B'B'B' (H303, H405)
Type 8									 B'B'B' (H213)* B'B'B'B'B' (H336)
Type 9									 B'B'B'B' (H384)*
Type 10									 B'B'B'B'B' (H339-1)*
Type 11									 B'B'B'B'B'B' (H536)*

FIG. 3. Repetitive DNA distribution in different types of B-chromosomes in *Prospero autumnale* complex. Individual used as source of chromosomes depicted in the figure is marked with asterisk. (A) Co-localization of 5S (red) and 35S rDNA (green), except for Type 3, second row, where 35S rDNA is in red (marked by star). (B) Co-localization of 5S rDNA (red) and satellite DNA *PaB6* repeats (green). Scale bar = 1 μ m.

B-chromosomes and repetitive DNA accumulation

In several species, Bs have been shown to possess repeat families shared with the standard complement as well as B-specific families (Langdon *et al.*, 2000; Dhar *et al.*, 2002; Marques *et al.*, 2012; Martis *et al.*, 2012). Large insertions of plastid and mitochondrial sequences have been detected in rye (Martis *et al.*, 2012). The B-chromosomes of *P. autumnale* share three repeat families with the standard complement - 5S and 35S rDNAs and satDNA *PaB6*. 5S and 35S rDNA are well conserved and present in all eukaryotes, but *PaB6* is specific to the genus *Prospero*. *PaB6* is evolutionarily very active and accompanies diversification of the basic cytotypes and the polyploids (Jang *et al.*, Chapters 2 and 3), but interestingly, levels of *PaB6* amplification in Bs did not correlate with that in the respective standard complement (Jang *et al.*, Chapter 2, 3).

Bs in the phylogenetically well defined cytotype AA are relatively uniform in structure and repetitive DNA content and distribution (Fig. 3). The AA cytotype probably became isolated from the B⁷ genome during a period of glaciation (Parker *et al.*, 1991; Jang *et al.*, 2013). This might have allowed fixation of only a single type of B in this cytotype (Parker *et al.*, 1991). Eight different B-types were found in B⁷ diploids and polyploids. The five B-types found exclusively in autopolyploids might have originated from the most common type by large-scale amplification of *PaB6*.

Irrespective of their genomic constitution, B-chromosomes in polyploids had higher amounts of *PaB6* and 5S rDNA repeats than those of diploids. Amplification of *PaB6* and 5S rDNA usually coincides albeit to a different extent. Thus, these data suggest a positive correlation between the extent of repeat amplification and ploidy level. Analyses of other plant groups containing B-chromosomes at different ploidy levels are needed, however, to allow general conclusions to be drawn.

The lack of meiotic pairing between Bs and the standard complement excludes recombination as a direct mechanism mediating the spread of repeats. A mechanism that has

been suggested to contribute to tandem repeat copy number increase and homogenization is via extrachromosomal circular DNAs (eccDNAs; Cohen *et al.*, 2008; Navrátilová *et al.*, 2008). eccDNAs with similarity to tandem repeats sequences (particularly 5S rDNA and satellite DNAs), shared by Bs and the standard complement in *Prospero*, have been found in several plant genomes (Cohen *et al.*, 2008). eccDNAs, then, may be involved in repeat amplification in *Prospero*.

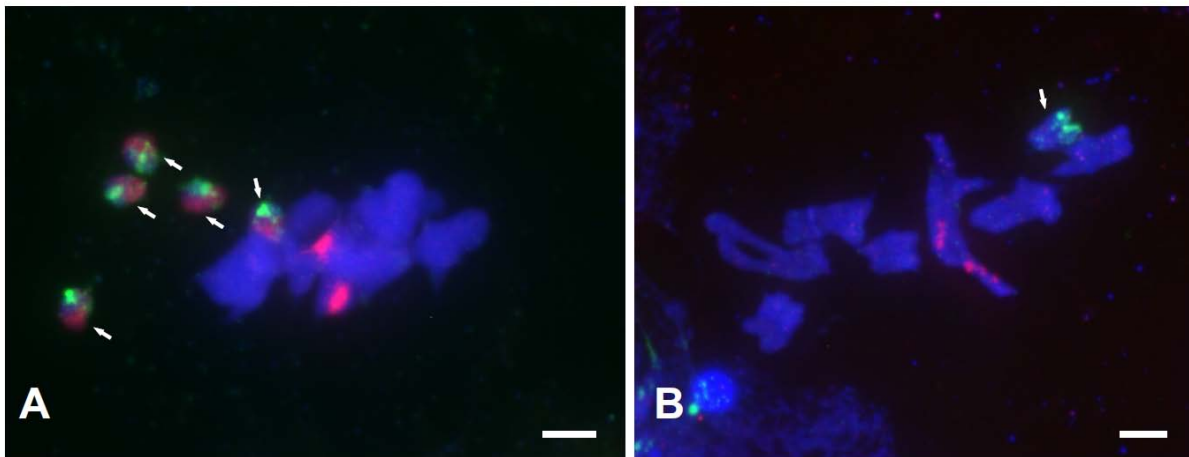


Fig. 4. Meiotic metaphase I of two *Prospero autumnale* individuals carrying B-chromosomes. (A) Cytotype B⁷B⁷ with five Bs (H412; 35S rDNA in red; PaB6 in green). (B) Cytotype B⁷B⁷ with two Bs (H415; 5S rDNA in red; PaB6 in green). Scale bar = 5 μm. Arrows indicate Bs.

On the origin of B-chromosomes in Prospero

The high levels of variation in structure and repeat composition of B-chromosomes of *P. autumnale* are suggestive of independent and multiple origins. Conversely, polymorphisms in structure and repeat content of B-chromosomes were also demonstrated in other taxa, even those possessing evolutionary stable Bs (Marques *et al.*, 2012). Polymorphisms, however, may be generated *de novo* from typical Bs. The observations on *Prospero*, however, are rather suggestive of ongoing, independent B-chromosome formation in this genus. *P. autumnale* is chromosomally extremely variable, with new diploid cytotypes evolving from an ancestral genome(s) on different evolutionary timescales (Jang *et al.*, 2013). This involved chromosomal fusions, inversions, and translocations accompanied by changes in the

repeatome. Proto B-chromosomes may have originated as a by-product of these extensive chromosomal changes. An additional level of chromosomal complexity in *Prospero* is introduced by polyploidy, and an ongoing origin of B-chromosomes has also been proposed in triploids of *Boechera holboellii* (Shrabel *et al.*, 2005).

Conclusions and outlook

The B-chromosomes of the chromosomally variable species complex of *P. autumnale* provide an excellent system for the analysis of many aspects of B-chromosome origin and evolution. The extent of variation of the repeat composition of B-chromosomes in *Prospero* - eleven types of Bs found in only 22 individuals - is extraordinarily high and fixes only the minimal level of variation. It is likely that other repeats will also contribute to this variation. Two other cytotype specific tandem repeats of *Prospero* (Weiss-Schneeweiss, Macas *et al.*, unpubl.), will be mapped, as well as major families of retroelements. These data should allow rigorous testing of the hypotheses of recurrent vs. single B-chromosome origin in *Prospero*. Large-scale meiotic analyses will address transmission to the next generation.

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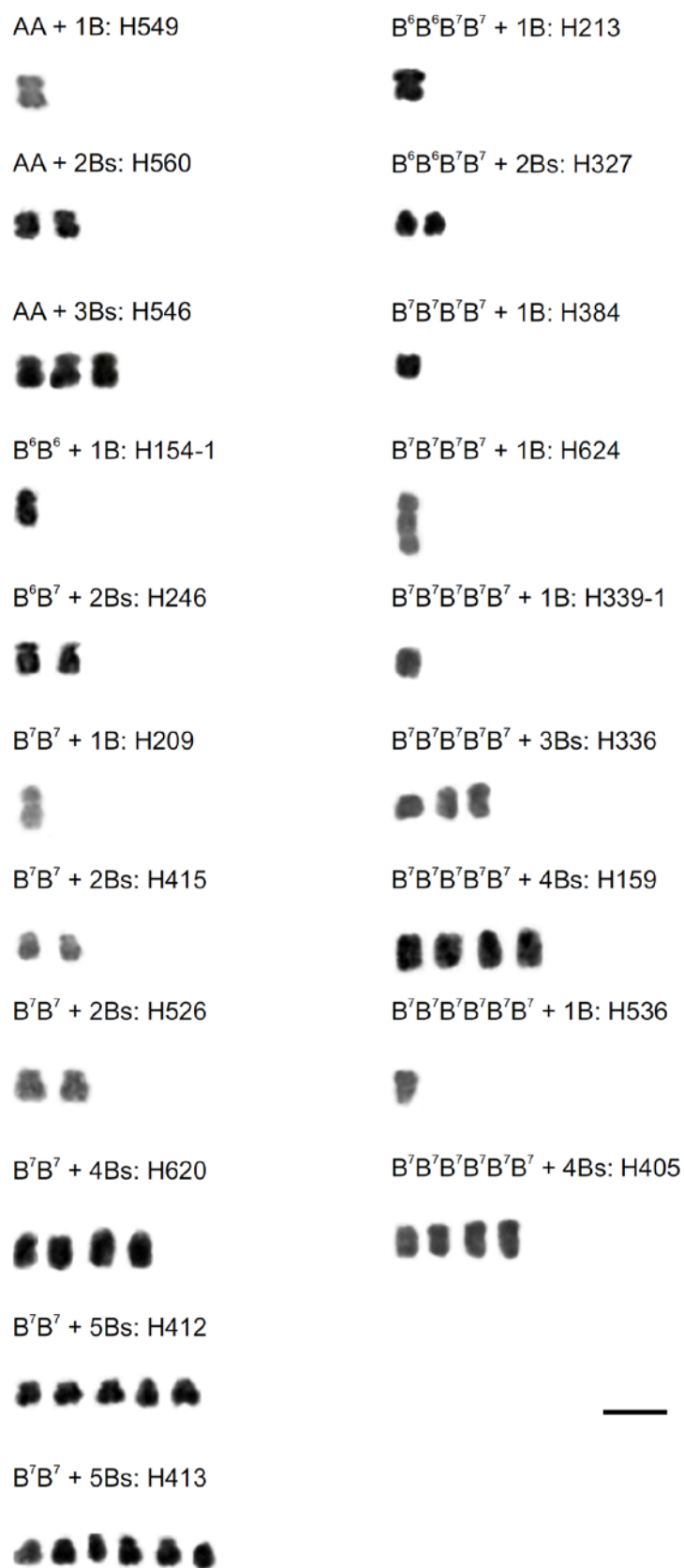
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SUPPLEMENTARY FIG. 1. Structure of B-chromosomes in all analyzed individuals. Scable bar = 5 μ m.



Appendix 1

Morphometric analysis of species and cytotypes in the genus *Prospero* (Hyacinthaceae)

Introduction

The genus *Prospero* Salisb. is usually referred to the family Hyacinthaceae, although it has recently been suggested that it should be placed within Asparagaceae (Govaerts et al., 2013). Govaerts et al. (2013) conclude that there are fourteen species in the genus, although it has been argued on genetic and cytological grounds that there are only two species and one cytologically diverse species complex (*P. hanburyi*, *P. obtusifolium*, and the *P. autumnale* complex; Jang et al., 2013). It is the purpose of this paper to assess morphological variation in the genus in the light of our understanding of chromosome evolution, and to establish whether any of the chromosomal variants, representing well defined evolutionary lineages show sufficient distinction for them to be raised to the level of species.

The specific rank of *P. hanburyi* ($2n = 14$) and *P. obtusifolium* ($2n = 8$) has never been challenged since they were first described, although both occur within the range of the *P. autumnale* complex. *P. hanburyi* is found in western Asia bordering the Mediterranean while *P. obtusifolium* grows in North Africa, Spain and the western Mediterranean islands. Neither species shows any karyotypic variation and both are karyotypically distinct and different from *P. autumnale* complex.

Prospero autumnale in the broad sense has been described as a single polymorphic complex including four basic cytotypes: AA (western part of Mediterranean region and Atlantic coasts, $2n = 2x = 14$), B⁵B⁵ (endemic to Libya, $2n = 2x = 10$), B⁶B⁶ (endemic to Crete, $2n = 2x = 12$), and B⁷B⁷ (widely distributed across the whole Mediterranean basin from Spain to Israel, $2n = 2x = 14$). This conclusion was based on a knowledge of chromosome number and size, location of the NOR regions (nucleolar organizer region or secondary constriction) and propensity to polyploidize (Hong, 1982; Ainsworth et al., 1983; Parker et al., 1991; Vaughan et al., 1993, 1997; Jang et al., 2013). Based on phylogenetic analyses of ITS sequence data of nuclear ribosomal DNA, three distinct clades have been proposed corresponding to A, B⁶, and paraphyletic B⁷ genome, with B⁵ deeply nested within the latter (Jang et al., 2013).

A combination of morphometric and genetic studies is necessary in understanding the delimitations of closely related taxa (Perný et al., 2005; Mandáková and Münzbergová, 2008; Sharma and Pandit, 2011; Bacchetta et al., 2012; Catalán et al., 2012; Jones et al., 2012). No taxonomic studies of the genus *Prospero*, however, have included comparative quantitative and qualitative morphological analysis, and none have combined these data with cytogenetic knowledge, which is crucial for delimitation of distinct genomic entities. Multivariate morphometric analyses have never previously been carried out to clarify the delimitation of the genus *Prospero* and to assess its species structure. The primary purpose of this study is, therefore, to (1) identify statistically significant morphological differences between individuals and cytotypes, (2) clarify the taxonomic confusion within *Prospero* using multivariate analysis.

Materials and methods

For this study, data of the morphology and cytology of 141 individuals of the genus *Prospero* were collected from wild-collected living plants held in a common botanical garden (University of Vienna). A phenetic study of these individuals was carried out based on 21 morphological characters (Table 1), using principle component analysis (PCA). *P. hanburyi* ($2n = 14$) and *P. obtusifolium* ($2n = 8$) were chromosomally constant and diploid but within the *P. autumnale* complex diploid and polyploid individuals with $2n = 10, 12, 14, 19, 20, 25, 26, 27, 28, 35, 42$ were included. Chromosome numbers and basic karyotypes for each individual were established as described in Jang et al. (2013). Diploid cytotype B⁵B⁵ has not been included in the analyses because the available bulbs did not flower and produced only few leaves.

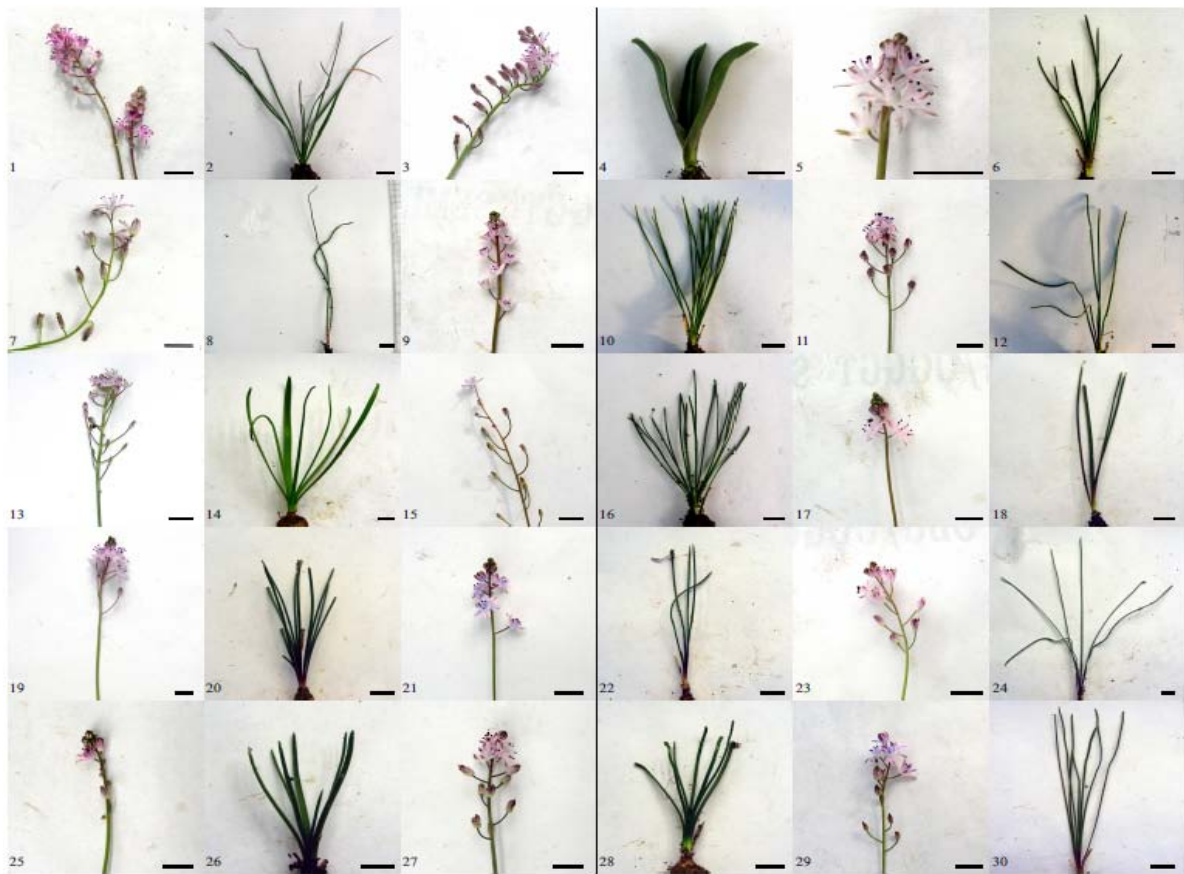


Fig. 1. Morphological features of *Prospero*. 1–2 *P. hanburyi*, 3–4 *P. obtusifolium*, 5–30 *P. autumnale* complex, 5–6 AA ($2n = 2x = 14$), 7–8 B^5B^5 ($2n = 2x = 10$), 9–10 B^6B^6 ($2n = 2x = 12$), 11–14 B^7B^7 ($2n = 2x = 14$), 15–16 $B^6B^6B^7$ ($2n = 3x = 20$), 17–18 AAB^7B^7 ($2n = 4x = 28$), 19–20 $B^6B^6B^7B^7$ ($2n = 4x = 26$), 21–22 $B^7B^7B^7B^7$ ($2n = 4x = 28$), 23–24 $B^7B^7B^7B^7B^7$ ($2n = 5x = 35$), 25–26 $AAB^7B^7B^7B^7$ ($2n = 6x = 42$), 27–28 $B^6B^6B^7B^7B^7B^7$ ($2n = 6x = 42$), 29–30 $B^7B^7B^7B^7B^7B^7$ ($2n = 6x = 42$).

Table 1. Morphological characters used for the morphometric analysis.

Number	Character	Codes
Vegetative characters		
1	Leaf length	cm
2	Leaf width	cm
3	Bulb length	cm
4	Bulb width	cm
5	Numer of leaves	Numbers
Floral characters		
6	Inflorescence length	cm
7	Number of inflorescences	Numbers
8	Pedicel length	cm
9	Number of flowers	Numbers
10	Tepal length	cm
11	Tepal width	cm
12	Filament length	mm
13	Filament width	mm
14	Anther length	mm
15	Anther width	mm
16	Ovary length	mm
17	Ovary width	mm
18	Style length	mm
19	Style width	mm
20	Ovule length	mm
21	Ovule width	mm

Results and Discussion

Despite the cytological complexity of *Prospero*, the plants show striking uniformity in floral and vegetative characters (Fig. 1). UPGMA cluster analysis of all morphological characters showed no clear pattern in the dendrogram (Fig. 2). However, box plots separate the two stable species *P. hanburyi* on style length (Fig. 3A) and *P. obtusifolium* on leaf width (Fig. 3B) from the *P. autumnale* complex.

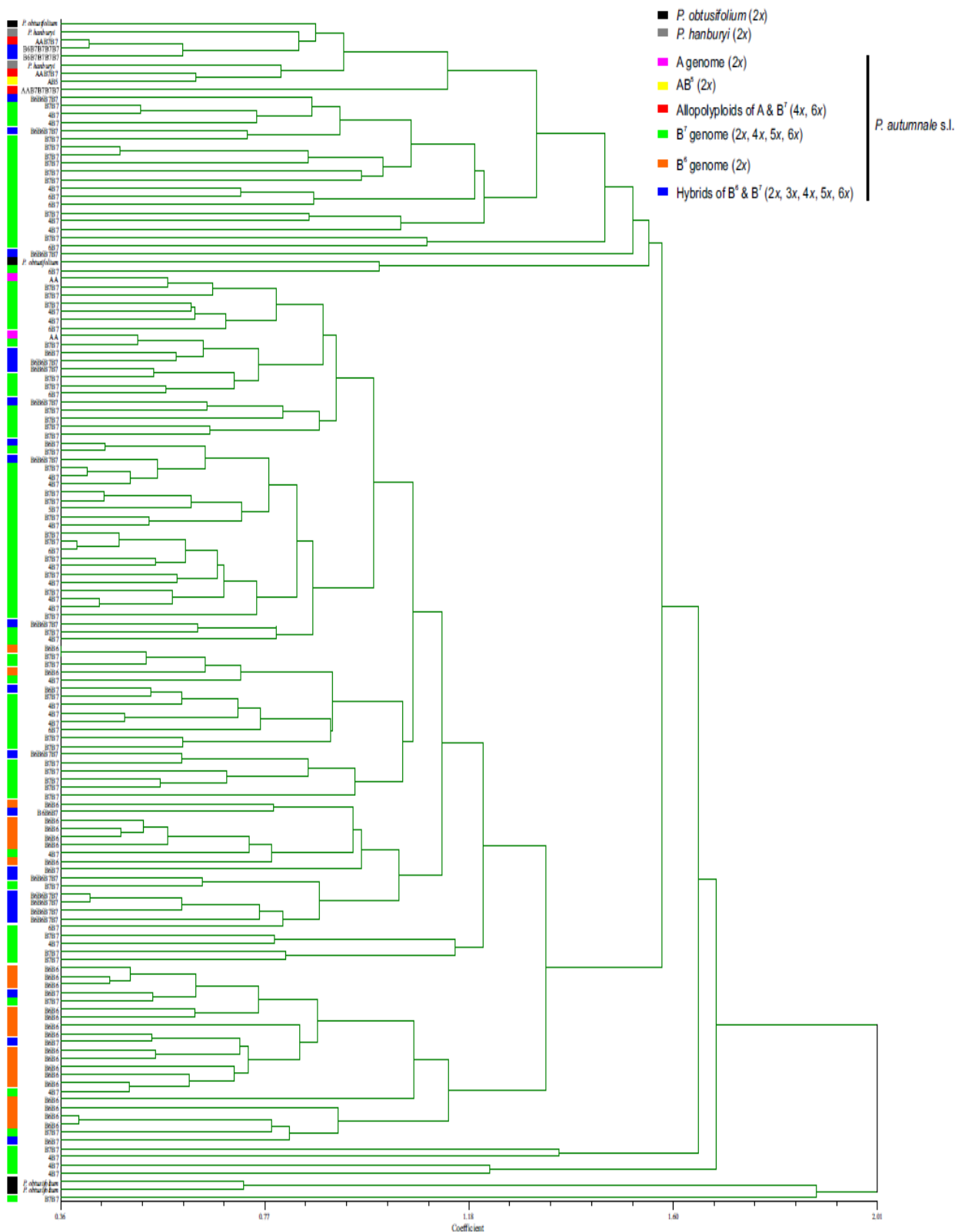


Fig. 2. Dendrogram illustrating the morphological relationships within the genus *Prospero* based on UPGMA clustering.

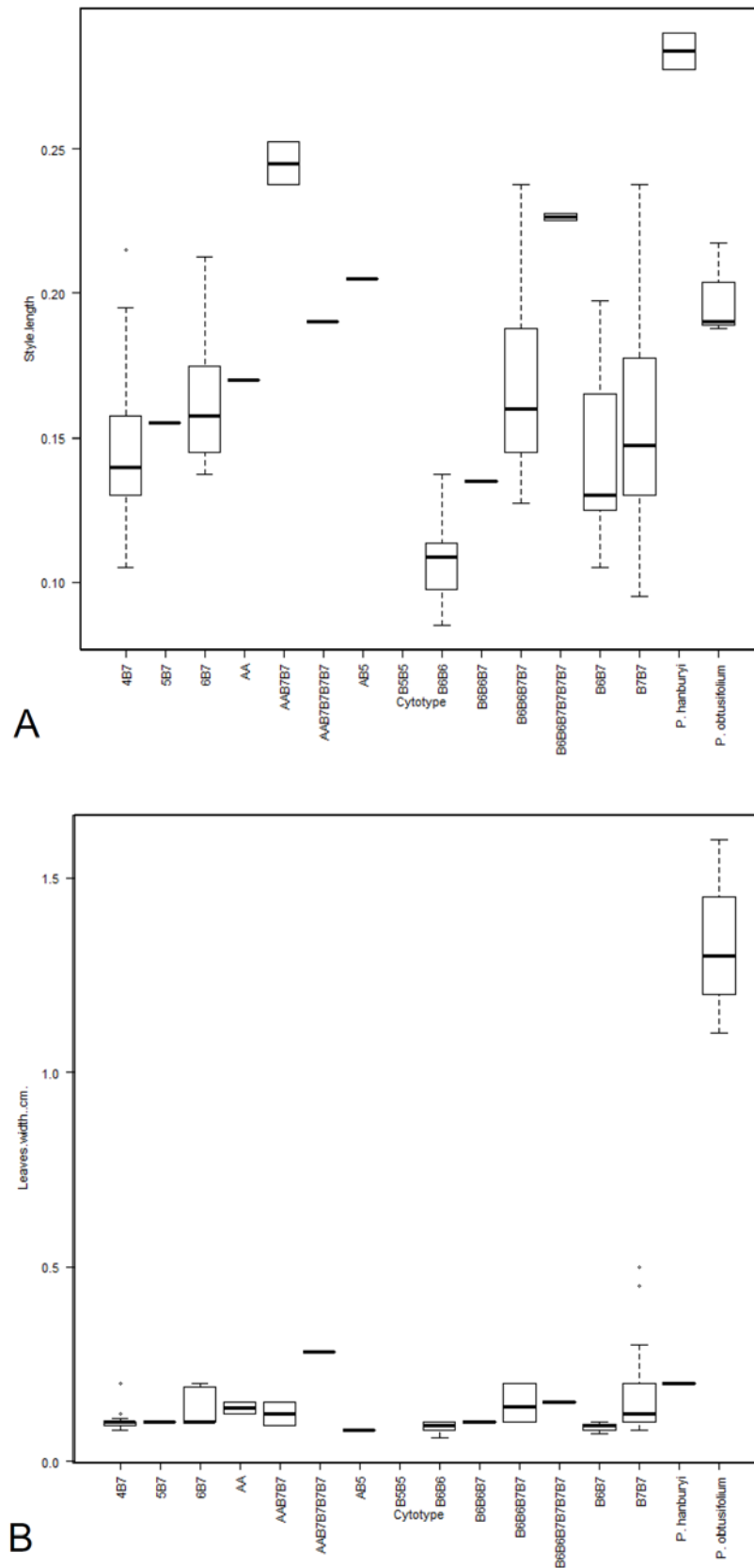


Fig. 3. Box plots of style length (A) and leaf width (B) quantitative characters included in the principal component analysis (PCA). No other groups could be separated on the basis of morphology.

The morphological PCA failed to separate the diploid cytotypes of *P. autumnale*, although the B⁶ genome shows slight clustering (Fig. 4A, circled). These plants are, however, interspersed with B⁷ (Fig. 4A). Speta (2000) referred to Cretan plants with thin leaves, a basic numbers of $x = 6$ and a large metacentric chromosome in the complement as *P. minimum*. This cannot be supported from the multivariate analysis. He also described three different endemic Cretan species, all with $2n = 14$ based on bulb, leaf, and flower size as *P. rhadamanthi*, *P. idaeum*, and *P. depressum*. Again, the PCA analysis, based on the same morphological characters, shows no clustering pattern in plants with the B⁷ genome. No other cytotypes can be distinguished from the main cluster by PCA analysis (Fig. 4A). The *P. autumnale* diploids, then, are continuously variable in morphology, making it impossible to identify cytotypes in natural population.

If autopolyploids and allopolyploids of B⁷, A and B⁶ genomes are included in the PCA together with the diploids, the dispersal is even more complicated and no nodes emerge (Fig. 4B). It is impossible not only to resolve diploids from higher ploidy levels but to identify any, even minute, diploid clustering any longer (Fig. 4B).

In conclusion, within genus *Prospero* it is possible to identify *P. hanburyi*, on the basis of style length, and *P. obtusifolium* on the basis of leaf width. Remarkably, no other groups emerge, irrespective of chromosome number or ploidy level differences. This then requires us to refer to the remaining plants as *P. autumnale*, a complex demonstrably in evolutionary flux.

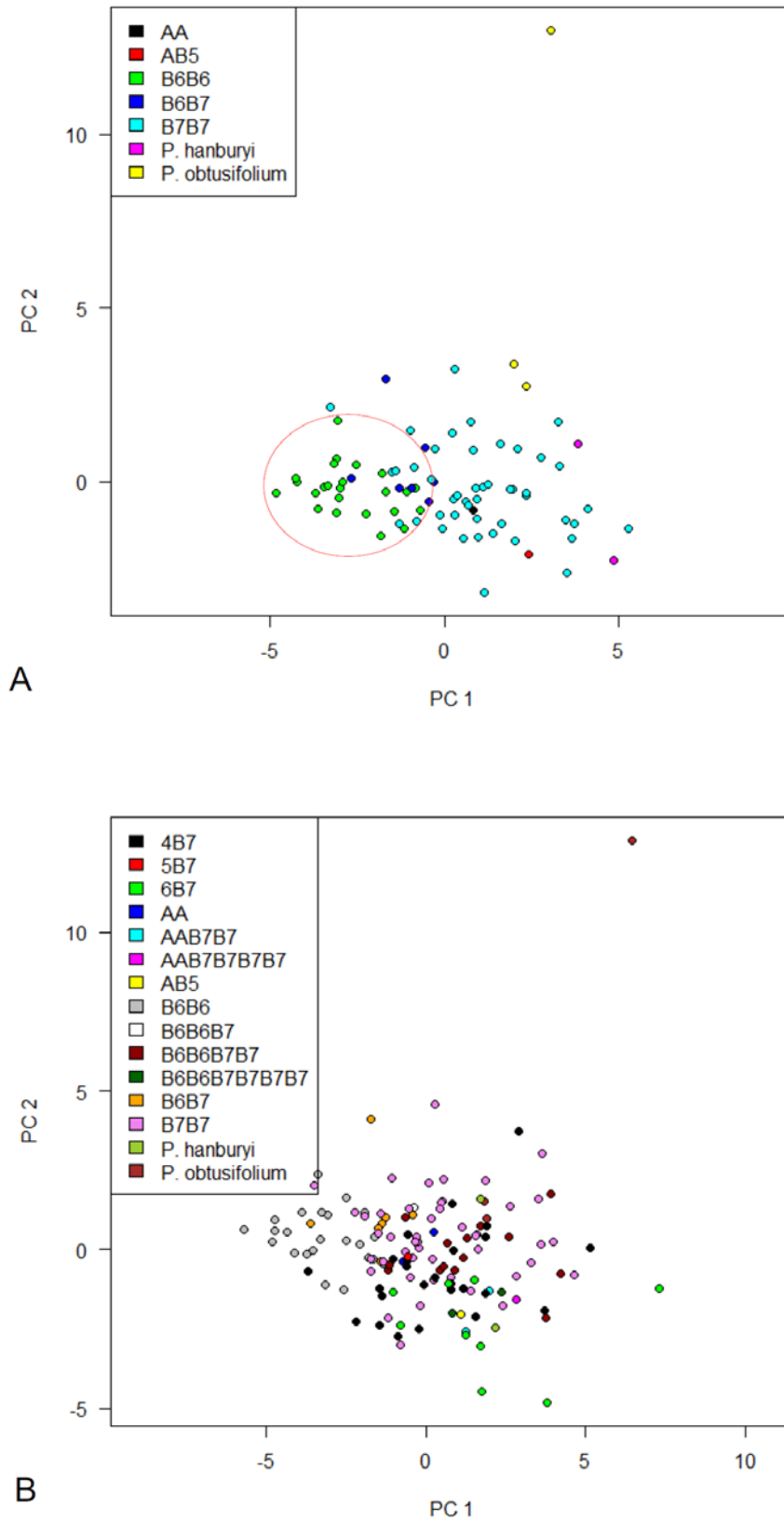


Fig. 4. Principal component analysis (PCA) of the genus *Prospero* based on morphological characters. A, PCA plot of diploids in the *P. autumnale* complex with *P. obtusifolium* and *P. hanburyi*; B, PCA plot of diploid homoploid hybrids and polyploids of the *P. autumnale* complex, with the other two species.

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Summary and conclusions

Angiosperms vary in rates of their genomic and chromosomal evolution (Weiss-Schneeweiss and Schneeweiss, 2013). Monocots are commonly recognized as more chromosomally variable than dicots, with several families of monocots experiencing highly elevated rates of rearrangements. One such system is in genus *Prospero* of family Hyacinthaceae (Ainsworth et al., 1983; Ebert et al., 1996; Vaughan et al., 1997). Genus *Prospero* encompasses three commonly recognized species, *P. obtusifolium* occurring in the western Mediterranean, *P. hanburyi* in the eastern Mediterranean, and the *Prospero autumnale* complex distributed over the whole Mediterranean region (Vaughan et al., 1997). While the first two species are chromosomally rather stable and known only from diploid level ($2n = 8$ and 14 , respectively), the *Prospero autumnale* complex exhibits remarkable levels of chromosomal variation. This variation is manifested in the presence of at least four basic diploid cytotypes, several genomic combinations of their polyploids, and a myriad of other chromosomal polymorphisms, including the presence of B-chromosomes and supernumerary chromosomal segments. Therefore genome evolution in all basic diploid and polyploid cytotypes of *Prospero* was addressed in attempt to understand the dynamics, mechanisms and directionality of chromosomal evolution.

Diploids

Although indistinguishable at the morphological level, the *Prospero autumnale* complex encompasses a four chromosomally variable diploid cytotypes (Ainsworth et al., 1983; Parker et al., 1991; Ebert, 1993; Ebert et al., 1996; Taylor, 1997; Vaughan et al., 1997; Jang et al., 2013). These cytotypes differ in chromosome number (AA , B^7B^7 , both $x = 7$; B^6B^6 , $x = 6$; B^5B^5 , $x = 5$), karyotype structure and genome size. One of the objectives of the current study was to establish whether these diploid cytotypes represented well-defined evolutionary lineages and which mechanisms were involved in their origin.

Unique combinations of numbers and distribution of three tandem repeats, 5S rDNA, 35S rDNA, and *PaB6* in chromosomes of all cytotypes implemented with genome size were sufficient to allow unambiguous identification of all diploid *Prospero* species and cytotypes. Karyotypes of *P. obtusifolium* and *P. hanburyi* were distinct from one another and from *P. autumnale* complex to an extent which did not allow direct comparisons. Conversely, high degree of homology among karyotypes of the four basic diploid cytotypes within *P. autumnale* complex enabled such comparisons. No variation within cytotypes was

encountered, except for the widespread B^7B^7 which included two distinct lineages, differing in 5S rDNA locus type and the extent of *PaB6* amplification. Interpretation of chromosomal variation in a phylogenetic context (ITS sequence comparisons) allowed inferences about the directionality of karyotype evolution to be made. The dysploidy series in *P. autumnale* earlier proposed to descend sequentially from $x = 7$ to 6 to 5 (Vaughan et al., 1997) was instead shown to result from independent chromosomal fusions, once from $x = 7$ to $x = 6$, and more recently from $x = 7$ to $x = 5$ (Jang et al., 2013). Fusion of chromosomes 6 and 7 resulted in a large submetacentric chromosome [$F_1(1-6/7)$], to give $x = 6$ (B^6B^6), while two other fusions, of chromosome 1 with 6 or 7 [$F^2(1-6/7)$], and of chromosome 3 with 7 or 6 [$F^3(3-6/7)$] resulted in $x = 5$ (B^5B^5). All diploid cytotypes possessed unique amounts satDNA *PaB6* in pericentromeric regions of some to all chromosomes. Highest levels of amplification of *PaB6* were observed in cytotypes B^6B^6 and B^5B^5 correlating with occurrence of fusions and other rearrangements.

Genomic consequences of polyploidy

The incidence of polyploidization with or without hybridization is a common phenomenon in many plant groups (Soltis et al., 2009; Weiss-Schneeweiss et al., 2013). Both auto- and allopolyploidization have been documented as driving forces of genome evolution in plants, but also in other organisms (Husband et al., 2013). In *Prospero*, polyploidy has been documented in all diploid cytotypes with the exception of the phylogenetically youngest B^5B^5 (Ainsworth et al., 1983; Ebert, 1993; Vaughan et al., 1993, 1997; Taylor, 1997; Speta, 2000; Hamouch et al., 2010). Autopolyploids occur only in cytotype B^7B^7 , while allopolyploids are produced from one of the following two parental genome combinations: (i) A and B^7 and (ii) B^6 and B^7 (Vaughan et al., 1997).

Detailed characterization of all diploid cytotypes provided tools and a framework in which to analyze polyploid genomes of *Prospero*. Analyses of auto- and allotetraploids ($B^7B^7B^7B^7$; AAB^7B^7 and $B^6B^6B^7B^7$) were performed to reveal the extent and dynamics of their chromosomal and genomic variation and elucidate their origin. These data allowed the inferences and correlations of parental genome divergence and dynamics of allopolyploid genomes (stability vs. lability).

A diverse array of experimental approaches, involving comparative cytogenetic analyses of the patterns and dynamics of three types of repetitive DNA (5S, 35S rDNA, satellite DNA *PaB6*), inference of molecular phylogenetic relationships based on both nuclear and plastid markers, and influence of affinities of parental genomes on meiotic processes and resulting genomic changes in polyploids allowed for better understanding of the evolutionary

significance of chromosomal changes on polyploid level. The detailed insight into the origin of polyploids, particularly in very complex allopolyploids of B⁶ and B⁷ genomic constitutions was possible.

Autotetraploids were shown to be genomically stable and additive compared to diploid progenitors. Allotetraploids varied in patterns of genome evolution depending on parental genomes affinities. Allotetraploids AAB⁷B⁷ were genomically stable, with homologous bivalent pairing, and consistent loss of the paternal 35S rDNA loci.

In contrast, allopolyploids of B⁶ and B⁷ origin were much more variable, but the variation was structured resulting from multiple cycles of hybridization. Four groups of genomically unique and restructured individuals were detected, varying in genome sizes, rDNA and satellite DNA loci numbers and localization. Primary crosses of genomically similar diploid B⁶B⁶ and B⁷B⁷ cytotypes produced genetically balanced allotetraploids possessing a range of chromosome numbers ($2n = 25\text{--}28$). These allotetraploids tolerated both homo- and homoeologous and mostly bivalent pairing. This was reflected in the dynamics of DNA repeat composition. Both 5S rDNA and *PaB6* increased their copy and signal numbers in primary allotetraploids. The numerical chromosome number variation of primary allotetraploids accompanied parental genomes reshuffling. Ultimately, only plants with $2n = 28$ successfully entered another cycle of hybridization with the second lineage of cytotype B⁷ and produced stable secondary allotetraploids (crosses of primary allotetraploids with B⁷ genome) which have further backcrosses to one of their parents or grandparents. Phylogenetic analysis of three plastid regions and nuclear ITS allowed maternal and paternal origin of most polyploids to be inferred enabling better interpretation of karyotypic variation.

Another level of chromosomal and genomic complexity in *Prospero* is introduced by the presence of supernumerary and dispensable genetic elements – B-chromosomes. Analyses of the structure of B-chromosomes in *Prospero* presented patterns and rates of accumulation of repetitive DNA families (5S, 35S rDNA, and satellite DNA *PaB6* loci) during B chromosome evolution. B-chromosomes were detected in all diploid cytotypes, except most-derived B⁵ genome. High levels of variation in copy number and distribution of the three types of tandem repeats were detected in B-chromosomes of the *Prospero autumnale* complex. B-chromosomes usually possessed 5S rDNA and *PaB6* repeats. Bs occurring in polyploid backgrounds were usually highly enriched in both of these repeats. Eleven different combinations of rDNA and *PaB6* distribution have been observed in Bs of the *P. autumnale* complex. Structural and genomic variation of B-chromosomes in chromosomally variable *Prospero autumnale* suggests their recurrent origin and ongoing evolution. Accumulation of

the tandem repeats might facilitate regular chromosome complement-independent pairing of Bs in *P. autumnale* complex, and allow their establishment in populations.

Very high levels of structured genomic and chromosomal variation in genus *Prospero*, and particularly in the *P. autumnale* species complex have never been explored in relation to the existing genus taxonomy. Analyses of morphological characters of multiple individuals of diploid and polyploid individuals of *Prospero* enabled the identification of *P. hanburyi*, on the basis of style length, and *P. obtusifolium* on the basis of leaf width. Additional taxonomic conclusions were, however, not permitted. Not only is it impossible to distinguish diploids from polyploids but also to identify diploid cytotypes clusters in *P. autumnale* complex. Thus, high levels of chromosomal variation contrast with morphological invariability in the *P. autumnale* complex. All results of this study suggest that diversification, and perhaps also speciation of genus *Prospero* might be, at least partly, driven by chromosomal restructuring. Thus, *Prospero* emerges as a model system in which to study the role of chromosomal change in plant diversification.

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Curriculum Vitae

Tae-Soo Jang

First name: Tae-Soo, Last name: Jang

Nationality: Republic of Korea

Education

- PhD Studies in Botany: since 2010 (University of Vienna, Vienna, Austria)

[Ph. D. Thesis: Chromosomal evolution in *Prospero autumnale* complex - supervisor: Ass. Prof. Dr. Hanna Schneeweiss, Priv.Do.]

- MSc in Biology: 2006 - 2008 (Kyung Hee University, Seoul, Republic of Korea)

[MSc Thesis: Systematics of the genus *Glechoma* L. and related genera (Nepetinae, Lamiaceae) - supervisor: Prof. Dr. Suk-Pyo Hong]

- B.Sc. in Biology: 1999 - 2006 (Kyung Hee University, Seoul, Republic of Korea)

[BSc Thesis: Sexual dimorphism of the terrestrial *Persicaria amphibian* (L.) S.F.Gray (Polygonaceae) in Swedish natural populations - supervisor: Prof. Dr. Suk-Pyo Hong]

Research Interests

Karyotype and genome evolution; polyploidy; repetitive DNA evolution; accessory genetic material (B-chromosomes and supernumerary segments - origin and evolution); molecular cytogenetics: FISH (fluorescence *in situ* hybridization) and GISH (genomic *in situ* hybridization).

Publications

- 1) Hong S-P, Song J-H, Jung E-H, Jeon Y-C, Lee W-J, **Jang T-S**, Moon HK. 2004. A study on the flora of Mt. Gwangduk (Kangwon Province). *Bulletin of Research Institute for Basic Sciences* **1**:175–196.
- 2) **Jang T-S**, Hong S-P. 2007. The taxonomic consideration of leaf epidermal microstructure in *Glechoma* L. (Nepetinae, Lamiaceae). *Korean Journal of Plant Taxonomy* **37**:239–254.
- 3) Kim S-Y, Kim C-S, Kim G-R, Kim J-K, Park S-H, **Jang T-S**, Lee W-K, Lee J-K. 2008. Chromosome numbers and karyotype analyses for 33 taxa of medicinal plants in Korea. *Korean Journal of Medicinal Crop Science* **16**:161–167.
- 4) Lee J-K, Park S-H, Ali MA, Park J-M, Kim G-R, Kim J-K, Lee C-Y, **Jang T-S**. 2009. Medicinal Plants of Korea. Seeds & Seedling Morphology. Creseed Publishing Co., Ltd.

- 5) Lee J-H, Kwon O-W, **Jang T-S**, Roh H-S, Hong S-P. 2010. The petiole anatomy of the genus *Spiraea* L. (Rosaceae) in Korea. *Korean Journal of Plant Taxonomy* **40**:16–26.
- 6) **Jang T-S**, Hong S-P. 2010. The nutlet morphology of the genus *Glechoma* L. (Lamiaceae) and its related taxa. *Korean Journal of Plant Taxonomy* **40**:50–58.
- 7) **Jang T-S**, Hong S-P. 2010. Systematic implications of pollen morphology in *Glechoma* L. and *Marmoritis* Benth. (Nepetinae, Lamiaceae). *Journal of Systematics and Evolution* **48**:464–473.
- 8) **Jang T-S**, Jeon Y-C, Hong S-P. 2010. Systematic implications of pollen morphology in *Elsholtzia* (Elsholtzieae-Lamiaceae). *Nordic Journal of Botany* **28**:746–755.
- 9) **Jang T-S**, Hong S-P. 2011. Gynodioecy and floral dimorphism of *Glechoma longituba* (Nakai) Kuprian. (Lamiaceae) in Korea. *Korean Journal of Plant Taxonomy* **41**:202–208.
- 10) **Jang T-S**, Emadzade K, Parker J, Temsch EM, Leitch AR, Speta F, Weiss-Schneeweiss H. 2013. Chromosomal diversification and karyotype evolution of diploids in the cytologically diverse genus *Prospero* (Hyacinthaceae). *BMC Evolutionary Biology* **13**:136.
- 11) Weiss-Schneeweiss H, Emadzade K, **Jang T-S**, Schneeweiss GM. 2013. Evolutionary consequences, constraints and potential of polyploidy in plants. Themed Issue of *Cytogenetics and Genome Research*: “Trends in polyploidy research in animals and plants” **140**:137–150.

Invited Talks

2012. Department of Biology, Faculty of Science, University of Split, Split, Croatia.

2013. Department of Plant Anatomy and Cytology, University of Silesia, Katowice, Poland.

Scientific Visits

24–30 October 2011: Charles University in Prague, Faculty of Science, Department of Botany, Prague, Czech Republic.

04–11 September 2012: University of Split, Faculty of Science, Department of Biology, Split, Croatia.

02–07 June 2013: Department of Plant Anatomy and Cytology, Silesian University, Katowice, Poland.

Participation in Conferences

2004. Korean Association of Biological Sciences. Seoul, Republic of Korea.

2006. Korean Association of Biological Sciences. Seoul, Republic of Korea.

2007. Korean Association of Biological Sciences. Seoul, Republic of Korea.

2008. Botany 2008. Vancouver, Canada.

2009. Korean Association of Biological Sciences. Daejeon, Republic of Korea.

2010. International Symposium “Biodiversity and Evolutionary Biology” of the German Botanical Society (DBG). Vienna, Austria.

2011. Korean Society of Plant Taxonomists. Seoul, Republic of Korea.

2012. International conference on polyploidy, hybridization and biodiversity (ICPHB 2012). Průhonice near Prague, Czech Republic.

2013. BioSyst.EU 2013. Vienna, Austria.

2013. Botany 2013. New Orleans, U.S.A.

Abstracts of oral contributions to conferences (* presenter)

Weiss-Schneeweiss H*, **Jang T-S**, Renny-Byfield S, Macas J, Emadzade K, Leitch AR, Parker J. 2010. Extent, origin and evolution of remarkable chromosomal variation in *Prospero autumnale* Speta (*Scilla autumnalis* L.; Hyacinthaceae): an interdisciplinary approach. *Society for Experimental Biology*, Prague, Czech Republic.

Weiss-Schneeweiss H*, Renny-Byfield S, **Jang T-S**, Macas J, Emadzade K, Leitch AR, Parker J, Speta F. 2010. Unparalleled chromosomal variation in monocot genus *Prospero* (Hyacinthaceae). *19th International Symposium “Biodiversity and Evolutionary Biology” of the German Botanical Society (DBG)*, Vienna, Austria.

Weiss-Schneeweiss H*, **Jang T-S**, Emadzade K, Macas J, Renny-Byfield S, Leitch AR, Parker J. 2010. Genome plasticity in *Prospero* (Hyacinthaceae). *International Workshop Structural and functional diversity of the eukaryotic genome*, Brno, Czech Republic.

Jang T-S*, Weiss-Schneeweiss H*. 2011. Chromosomally dynamic genus *Prospero*: evolution of selected repetitive DNA types. *PolygenPhD meeting*, London, U.K.

Weiss-Schneeweiss H*, **Jang T-S**, Emadzade K, Macas J, Kovařík A, Leitch AR, Renny-Byfield S, Speta F, Parker J. 2012. Genome plasticity of polyploids in the chromosomally variable *Prospero autumnale* complex (Hyacinthaceae) contrasts with the genome stability of diploids. *International Conference on Polyploidy, Hybridization and Biodiversity (ICPHB 2012)*, Průhonice, Czech Republic.

Ataei N*, Valizadeh J, Temsch EM, **Jang T-S**, Endl E, Dolf A, Schneeweiss G, Garcia MA, Ballmann M, Wicke S, Quandt D, Weiss-Schneeweiss H. 2012. Evolutionary trends in non-photosynthetic parasitic *Cistanche* (Orobanchaceae) inferred from karyological data. *21st International Symposium “Biodiversity and Evolutionary Biology” of the German Botanical Society (DBG)*, Mainz, Germany.

Jang T-S*, Emadzade K, Temsch EM, Parker J, Weiss-Schneeweiss H. 2013. Evolution of genomes of allopolyploids is more dynamic than of diploid homoploid hybrids. *BioSyst.EU 2013, Global systematic*, Vienna, Austria.

Emadzade K*, **Jang T-S**, Parker J, Weiss-Schneeweiss H. 2013. Chromosomal evolution and taxa diversification in *Prospero* (Hyacinthaceae). *BioSyst.EU 2013, Global systematic*, Vienna, Austria.

Jang T-S*, Parker J, Weiss-Schneeweiss H. 2013. B-chromosomes in *Prospero autumnale* complex (Hyacinthaceae): structural polymorphisms and distinct repeat composition suggest their recurrent origin and ongoing evolution. *Botany 2013*, New Orleans, U.S.A.

Abstracts of poster contributions to conferences (* presenter)

Moon H-K*, **Jang T-S**, Hong S-P. 2004. Sexual dimorphism of the terrestrial *Persicaria amphibian* (L.) S.F.Gray (Polygonaceae) in Swedish natural populations. *The 59th annual meeting of the Korean Association of Biological Sciences*, Seoul, Republic of Korea.

Jang T-S*, Hong S-P. 2006. Evidence of Gynodioecy in *Agastache rugosa* (Fish.&Mey.) Kuntze (Lamiaceae). *The 61st annual meeting of the Korean Association of Biological Sciences*, Seoul, Republic of Korea.

Jang T-S*, Han M-J, Hong S-P. 2007. Gynodioecy and floral dimorphism of *Glechoma hederacea* var. *longituba* Nakai (Lamiaceae) in the Korean natural populations. *The 62nd annual meeting of the Korean Association of Biological Sciences*, Seoul, Republic of Korea.

Jang T-S*, Lee J-K, Hong S-P. 2007. Pollen morphology and ITS analysis of the genus *Glechoma* L. and related genera (Nepetinae-Lamiaceae). *The 62nd annual meeting of the Korean Association of Biological Sciences*, Seoul, Republic of Korea.

Jang T-S*, Hong S-P. 2008. Systematic implication of floral micromorphology in *Glechoma* and related genera (Nepetinae-Lamiaceae). *Botany 2008*, Vancouver, Canada.

Kim S-Y*, Kim C-S, Koh J-G, Tho J-H, Kim G-R, Kim J-K, Park S-H, **Jang T-S**, Lee W-K, Lee J-K. 2008. Chromosomes of endemic plants to Korea. *Botany 2008*, Vancouver, Canada.

Lee J-H*, Kwon O-W, **Jang T-S**, Roh H-S, Hong S-P. 2009. The petiole anatomy of the genus *Spiraea* L. (Rosaceae) in Korea and its taxonomic evaluation. *The 64th annual meeting of the Korean Association of Biological Sciences*, Daejeon, Republic of Korea.

Jang T-S*, Emadzade K, Renny-Byfield S, Leitch AR, Parker J, Speta F, Weiss-Schneeweiss H. 2010. Chromosome diversification and evolution in the *Prospero autumnale* complex (Hyacinthaceae). *19th International Symposium "Biodiversity and Evolutionary Biology" of the German Botanical Society (DBG)*, Vienna, Austria.

Jang T-S*, Emadzade K, Temsch E, Macas J, Leitch AR, Parker J, Speta F, Weiss-Schneeweiss H. 2011. Karyotype analysis of diploid and polyploidy species of the *Prospero* (Hyacinthaceae). *43rd Symposium, The Korean Society of Plant Taxonomists*, Seoul, Republic of Korea.

Jang T-S*, Emadzade K, Parker J, Speta F, Leitch AR, Fay M, Weiss-Schneeweiss H. 2012. Evolution of ribosomal DNA loci in polyploids of the *Prospero autumnale* complex (Hyacinthaceae). *International Conference on Polyploidy, Hybridization and Biodiversity (ICPHB 2012)*, Průhonice, Czech Republic.

- Emadzade K*, **Jang T-S**, Macas J, Kovařík A, Weiss-Schneeweiss H. 2012. Evolutionary dynamics of tandemly repeated satellite DNA *PaB6* in chromosomally variable *Prospero autumnale* polyploidy complex (Hyacinthaceae). *International Conference on Polyploidy, Hybridization and Biodiversity (ICPHB 2012)*, Průhonice, Czech Republic.
- Temsch EM*, **Jang T-S**, Lučanová M, Parker J, Speta F, Weiss-Schneeweiss H. 2012. Genome size variation in *Prospero* (Hyacinthaceae). *International Conference on Polyploidy, Hybridization and Biodiversity (ICPHB 2012)*, Průhonice, Czech Republic.