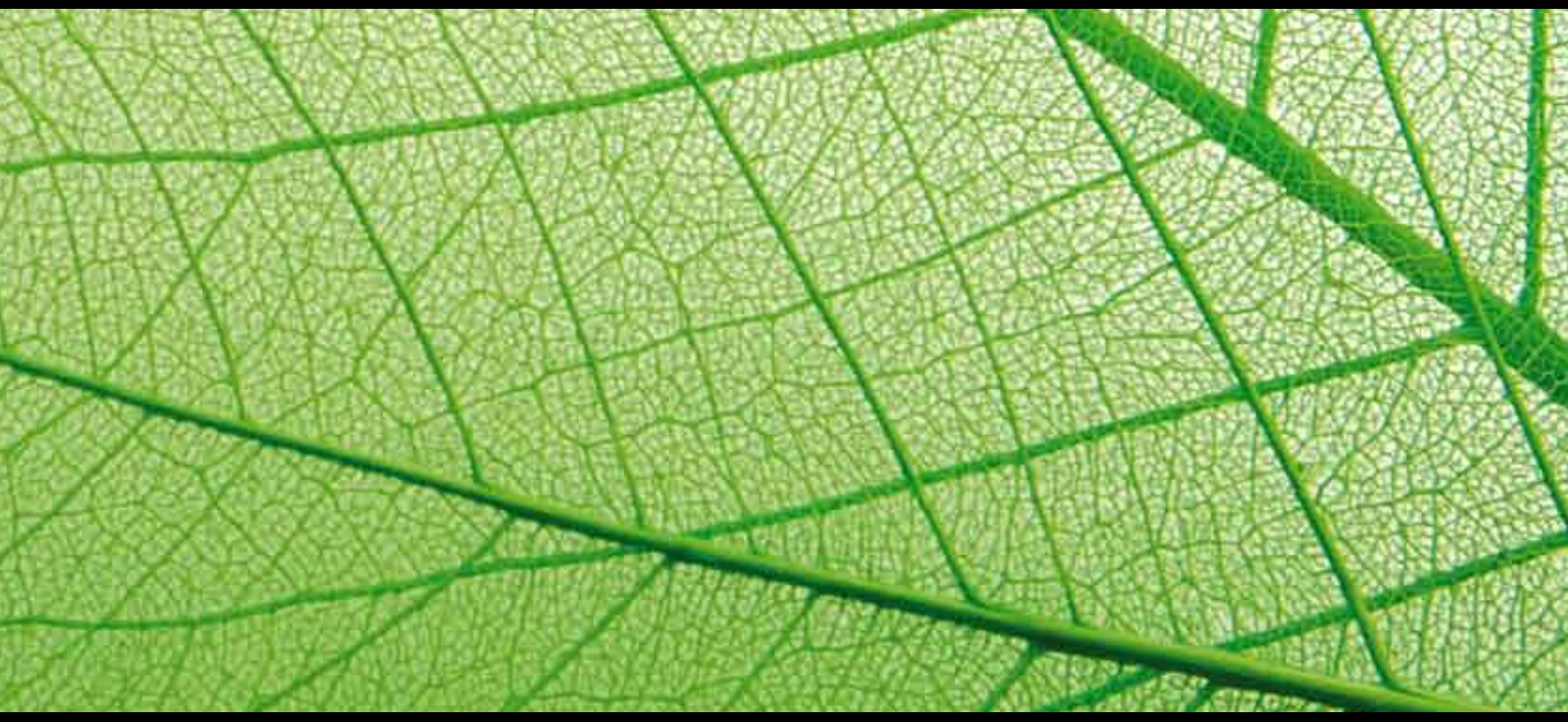
Journal of Botany

Genome Size

Guest Editors: Johann Greilhuber, Jaroslav Doležel, Ilia J. Leitch, João Loureiro, and Jan Suda



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Editorial Board

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 946138, 4 pages
doi:10.1155/2010/946138

Editorial

Genome Size

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Received 6 June 2010; Accepted 6 June 2010

The beginning of the third millennium has been an exciting time for biologists and geneticists in particular, as it is now possible to read DNA sequences at rates equivalent to a hundred human genomes per week and identify, in a high-throughput manner, differences between genomic loci as small as a single base pair. This rapid progress in genomics has led to the advent of metagenomics, which delivers genomic sequences of organisms occupying certain environments, and population genomics, which focuses on the genomes of many individuals to unravel patterns of neutral and adaptive variation in genomes. Such advances continue to dramatically improve our understanding of the structure of genetic information, its evolution and function, and provide us with the knowledge and tools that have the potential to revolutionize human healthcare, biotechnology, agriculture, and environmental protection.

While we can pinpoint minute changes in DNA sequences, identify chemical modifications of nuclear bases, and link these changes to the phenotype, we continue to be puzzled by the large variation in the size of the genome itself, of which there seems to be no rational explanation. Nevertheless, in seeking to understand the biological basis of this diversity and to understand its adaptive value, researchers have long searched for ecological, evolutionary, and taxonomic interpretations. While early observations that the amount of nuclear DNA did not correlate with organismal complexity were referred to as the "C-value paradox" [1], this was renamed the "C-value enigma" following the realization that much of the variation was due to repetitive DNA [2]. This change in terminology reflected the fact that whilst the basis of the variation in genome size was now understood (i.e., difference in amount of repetitive DNA), there were still many unanswered questions. Indeed, even today, with the mechanisms leading to genome size variation appearing to be well understood, the forces which drive this variation and determine the size of nuclear genome remain puzzling and there is no doubt that the "C-value problem" will stay with us for many years to come.

The observation of the immense variation in genome size raised the quest for biological correlates, stability versus plasticity, function versus effect, and selective significance versus inertness. Various hypotheses were put forward, with the "nucleotype hypothesis" by M. D. Bennett [3] playing a seminal role. He proposed that the DNA of an organism could influence its phenotype in two distinct ways, first through its informational content (genotype), and secondly, through the physical effects of its mass and volume (nucleotype) which could impose absolute limits on the range of phenotypes expressed by genic control. Thus biologists learnt that in addition to the environment and genotype, which influence the phenotype and hence adaptation and niche occupation, the nucleotype could also influence the phenotype largely through effects at the nuclear level such as cell size and cell cycle time [4].

An important prerequisite to study the biological role of genome size and its evolutionary changes was the availability of a sufficient amount of data on genome size for representative taxa [5]. The development of a "*Plant DNA C-values database*" by M. D. Bennett and I. J. Leitch was invaluable in this regard [6] as it provided a lighthouse for those studying genome size variation in plants. Since its launch in 2001, it has been used extensively to search for genome size, to find enough data to describe changes in genome size during evolution and speciation as well as to develop and test hypotheses on the biological significance of genome size variation. Currently the database contains genome size data for 5152 plant species (4429 angiosperms, 207 gymnosperms, 87 pteridophytes, 176 bryophytes, and 253 algae) compiled from over 500 original reference sources with a further update of over 1800 species of angiosperms expected by the end of 2010. On average the database receives over 2000 hits per month.

The first generation of researchers quantifying DNA amounts struggled with the lack of suitable approaches and instruments and largely used biochemical methods, which produced average values from many cells. Progress was made by the introduction of Feulgen microspectrophotometry, which provided a tool to measure DNA amounts in single cells. This method was widely adopted and led to the generation of genome size data for a vast number of species. The spectrum of methods suitable for plants expanded considerably in 1983, when D. W. Galbraith et al. [7] developed a protocol for measuring DNA content by flow cytometry. The method was shown to be convenient and rapid and has enabled large-scale studies to be undertaken, the scope of which would have been inconceivable using other methods. Flow cytometry is now almost exclusively used in botanical genome size research, not only because of the superiority of the method but also as a result of the increasing availability of affordable instruments. This contrasts with the situation with Feulgen densitometry where the old cytophotometer systems are now technically obsolete. It should be noted that while image cytometry has the potential to replace microdensitometry, to date this method has not been used extensively in plant sciences.

While acknowledging the dominance of flow cytometry, it should be noted that Feulgen densitometry still remains an important tool, for example to solve some methodological problems as described in this issue [8]. While there is still a need to improve DNA flow cytometry methods to enable reliable data on DNA amounts to be generated, current evidence indicates that this technique will continue to be instrumental in expanding research on genome size, making it a prosperous research area. But no matter what methods will be used to estimate the size of nuclear genomes, it seems safe to predict that future studies will increasingly incorporate tools of molecular biology and genomics with the hope of unravelling one of the many secrets of Mother Nature.

As the guest editors of this thematic issue of Journal of Botany, we have great pleasure in introducing sixteen highly valuable and stimulating reviews and original investigations. The topics range from methodological innovations for measuring nuclear DNA amount to investigations on the nucleotype theory and evolutionary phenomena at the quantitative and molecular level. Although the nucleotype theory may answer many questions as to the biological role of genome size, the evolution of genome size still has facets which are poorly understood, and indeed it seems likely that additional factors will be uncovered of which we are currently unaware.

The body of papers that constitute this special issue is led by the focal review of I. J. Leitch et al. (Kew and London, UK; and New Haven, Connecticut, USA) on "*Genome size dynamics and evolution in monocots*" [9]. This paper provides a detailed analysis of the physical genomic characters and genome size diversification across all monocot orders, conducted within a stringent phylogenetic context. It becomes clear that different monocot orders have followed distinct modes of genome size and chromosome evolution, and that several major increases and decreases have occurred.

J. M. Beaulieu et al. (New Haven, Connecticut; Durham, North Carolina, USA; and Kew, UK) use statistical modelling to analyse the effect of growth form on the tempo of genome size evolution in angiosperms, using the monocots and Fabaceae as target groups [10]. Rates of genome size evolution were found to be dependent on growth form with woody taxa evolving slower than herbaceous ones. This difference likely reflects, in part, the longer generation times in woody angiosperms.

In a timely and in-depth review, C. E. Grover and J. F. Wendel (Ames, Iowa, USA) analyse the various mechanisms of accumulation and removal of transposable DNA elements in the nuclear genome, which lead to the enormous variation observed, including an overview of the epigenetic controls and the population level processes which may operate [11].

T. Eilam et al. (Tel Aviv, Israel; Rehovot, Israel) review the exciting findings of rapid genome downsizing in natural and synthetic allopolyploids and diploidized autopolyploids in the Triticeae, which is in contrast to the additive genome sizes typically found in autopolyploids [12].

Extreme C-values are of special interest to flow cytometrists and researchers in genome size. B. J. M. Zonneveld (Leiden, The Netherlands) reports the unexpected discovery of new upper extremes in angiosperm genome size and corrects older values measured using classical Feulgen scanning densitometry [13]. Not unexpectedly, these new extremes are found in the monocot family Melanthiaceae and the eudicot family Viscaceae.

The nucleotype theory and ecological adaptation are the topic or at least an essential focus of four investigations.

M. H. Hoffmann et al. (Gatersleben and Halle-Wittenberg, Germany) present the results of a comparative study looking at genome size and chromosome number variation, phylogenetic relationships, and differences in developmental rates and reproductive success in *Arabidopsis* and cooccurring *Olimarabidopsis pumila, Arabis montbretiana,* and *A. auriculata* (Brassicaceae) from Uzbekistan [14]. They observed correlations between 1Cx-values and developmental rates and various phenological characters.

C. A. Knight et al. (San Louis Obispo, California, USA; Tartu, Estonia; and New Haven, Connecticut, USA) investigate the relationship between pollen grain size and

C-value across a phylogenetically broad sample of species and find no significant global correlation (except, a big split between gymnosperms and angiosperms) [15]. Their study highlights the dangers of extrapolating from small-scale analyses, which have suggested that genome size and pollen size were positively correlated, and hence demonstrates that pollen size cannot be used for estimating genome size of ancient taxa.

A. Gruner et al. (San Louis Obispo, California, USA) present the first results from a study aimed at investigating the relationship between genome size and apical root meristem growth rates in eight species of eudicots [16]. Using a novel approach involving time lapse microscopic image analysis they found that root growth was negatively correlated with genome size. This observation has a strong explanatory potential for the prevalence of small genomes in angiosperms.

E. M. Temsch et al. (Vienna, Austria) reappraise a recently published paper on the differential survival of plant species under conditions of heavy metal pollution [17]. The finding that increasing soil pollution with lead along a gradient goes parallel with the loss of species having larger genomes is fully confirmed by this new study, in which flow cytometry was used instead of DNA image densitometry.

Plant biosystematics has enormously profited from the introduction of nuclear DNA content measurement methods and in particular flow cytometry and is a dominating topic within the genome size discipline. Five papers of this special issue of Journal of Botany fall closely within this topic.

O. Hidalgo et al. (Athens, Ohio, USA; Montpellier, France; Barcelona, Spain; Kew, UK; and Barcelona, Spain) present the first large-scale genome size study in 36 species of Valerianaceae, backed by a molecular phylogeny and many chromosome counts [18]. A more than 20-fold variation in C-values, from an extremely low 0.4 pg to 8.32 pg/2C, is reported. Changes in the basic chromosome number and genome size characterize major morphological shifts in the evolutionary history of the family.

M. Höfer (Dresden, Germany) and A. Meister (Gatersleben, Germany) provide a nearly complete overview of genome sizes in cultivated and wild apple, 26 primary species and 20 hybrid species, and show correlations of genome size with geographical distribution and taxonomic grouping, as well as genome downsizing in some of the intraspecific polyploids [19].

B. J. M. Zonneveld (Leiden, The Netherlands) presents a complete dataset on *Hepatica* (Ranunculaceae) and demonstrates that contrary to published C-values, polyploid taxa of this genus have downsized their genomes [20].

K. Anamthawat-Jónsson et al. (Reykjavik, Iceland; Vienna, Austria) report on studies investigating the morphology, ploidy, introgression, and genome size (using both Feulgen densitometry and flow cytometry) in Icelandic birch species *Betula nana*, *B. pubescens,* and hybrids [8]. The exceptional constancy of the monoploid genome size at all three levels of ploidy is surprising in view of the different taxonomic position of the parental species and otherwise frequent occurrence of downsized genomes in polyploids and hybrids.

Endopolyploidy is an important phenomenon of tissue differentiation and has been widely investigated in angiosperms, but seldom in the bryophytes.

J. D. Bainard and S. G. Newmaster (Guelph, Ontario, Canada) address this gap by presenting the first study investigating the prevalence of endopolyploidy in bryophytes [21]. Endopolyploidy was observed in all of the moss species studied, with the exception of those belonging to the genus *Sphagnum*. Endopolyploidy was also noted to be absent in liverworts.

Methodological innovations in flow cytometry of plants are of the upmost importance and are the focus of two papers of the special issue and play a role also in a third one.

I. Jedrzejczyk and E. Sliwinska (Bydgoszcz, Poland) compare the performance of flow cytometry methodologies for the proper isolation of nuclei from leaves and seeds in 11 species of Rosaceae, a family known to be technically difficult because of the prevalence of secondary compounds in almost all tissues [22]. The authors find that seeds are the material of choice for genome size studies rather than leaves because of the absence of DNA staining inhibitors.

T. C. R. Silva et al. (Viçosa, Brazil) tackle a long-standing and hitherto unsolved question, that is, is fixed root tip material suitable for flow cytometry studies. They present a protocol focused on cell cycle studies [23]. Apart from this application, there is much still to be explored in the potential of this method for utilizing fixed and stored plant material collected in the wild.

K. Anamthawat-Jónsson et al. (see above) on Icelandic birch are mentioned once again here as their paper reports that decorticated dormant twigs (harvested in winter) are suitable vegetative material (and probably the only type of material of possible use) for flow cytometric ploidy and genome size determination in *Betula*, a woody plant genus known to be problematic for genome size studies due to its high phenolic content [8].

It is a pleasant duty to express our deepest thanks to the authors for accepting our invitation, their willingness to share their knowledge and research results, and for timely submission of authoritative reviews and original research articles. We appreciate the time they devoted to this endeavour and we hope that together we have created a useful resource for all interested in plant genome size. We wish you a nice and inspiring reading.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 862516, 18 pages
doi:10.1155/2010/862516

Review Article

Genome Size Dynamics and Evolution in Monocots

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Received 7 January 2010; Accepted 8 March 2010

Academic Editor: Johann Greilhuber

Monocot genomic diversity includes striking variation at many levels. This paper compares various genomic characters (e.g., range of chromosome numbers and ploidy levels, occurrence of endopolyploidy, GC content, chromosome packaging and organization, genome size) between monocots and the remaining angiosperms to discern just how distinctive monocot genomes are. One of the most notable features of monocots is their wide range and diversity of genome sizes, including the species with the largest genome so far reported in plants. This genomic character is analysed in greater detail, within a phylogenetic context. By surveying available genome size and chromosome data it is apparent that different monocot orders follow distinctive modes of genome size and chromosome evolution. Further insights into genome size-evolution and dynamics were obtained using statistical modelling approaches to reconstruct the ancestral genome size at key nodes across the monocot phylogenetic tree. Such approaches reveal that while the ancestral genome size of all monocots was small (1C = 1.9 pg), there have been several major increases and decreases during monocot evolution. In addition, notable increases in the rates of genome size-evolution were found in Asparagales and Poales compared with other monocot lineages.

1. Introduction: How Distinctive Are Monocot Genomes?

Monocotyledons (monocots) comprise c. 25% of all angiosperms and are a remarkably variable group with species found growing on all continents and in all habitats. They were first distinguished from other angiosperms by the presence of a single cotyledon [1] and they have since been shown to be strongly supported as sister to the eudicots + *Ceratophyllum* clade ([2] plus others).

At the genomic level, monocots are remarkably diverse, with striking variation at many levels ranging from gene sequences through to the number of chromosomes per genome, the number of genomes (ploidy), and the amount of DNA per genome (genome size). Yet how different are monocot genomes from other angiosperms? In this post-genomics era of large-scale sequencing and comparative analysis, the availability of large amounts of sequence information together with increasing amounts of more traditional cytological data provides new insights into this question.

This paper reviews available data to highlight some of the similarities and differences between monocots and the remaining angiosperms that have been revealed. One of the most striking features of monocots is their wide range of genome sizes, and this genomic character is analysed in greater detail to examine the diversity and dynamics of genome-size evolution within monocots.

2. Comparisons between the Genomes of Monocots with Other Angiosperms

Surveys of the literature and online databases have revealed that many aspects of monocot genomes are generally similar to other angiosperms.

2.1. Range of Chromosome Numbers. The minimum and maximum chromosome numbers so far reported for monocots and eudicots are similar. Both groups contain species with $2n = 4$ (four monocots and two eudicots) [6, 7], and

the highest number so far recorded is $2n$ = c. 600 for the monocot palm, *Voanioala gerardii* (Arecaceae) [8, 9], and $2n$ = c. 640 in the eudicot stonecrop *Sedum suaveolens* (Crassulaceae) [10].

2.2. Occurrence of Polyploidy and Maximum Ploidy Levels. In both monocots and eudicots 70%–80% of species are estimated to be cytological polyploids, suggesting similar propensities in each group to undergo polyploidization [11, 12]. There is also molecular evidence of ancient whole genome duplications not only at the base of both monocot and eudicot lineages but also in *Nuphar*, a member of an early diverging angiosperm lineage (Nymphaeaceae). This suggests that most if not all angiosperms retain evidence of polyploidy in their evolutionary history [13, 14]. Nevertheless, the maximum number of whole genome duplications so far reported is estimated to be only c. 38× in *Poa literosa* ($2n$ = c. 266) [15] and *Voanioala gerardii* [16] compared with 80× in *Sedum suaveolens* [10] perhaps pointing to differences between monocots and eudicots in the maximum possible number of polyploidy cycles. However, the reduction of chromosome numbers through dysploidy is a common mode of chromosome evolution in many groups which will obscure the signature of polyploidy over time. Thus, whether the observed differences in maximum ploidy levels reflect biologically different propensities to undergo polyploidy and/or dysploid reductions in monocots and eudicots is currently unknown.

2.3. Endopolyploidy. Endopolyploidy, the occurrence of elevated ploidy within cells of an organism arising either by endoreduplication or endomitosis [17], has been widely documented in angiosperms. However, surveys examining its occurrence in different families suggest that there is no significant difference between monocots and other angiosperms. For example, in a study of 49 species from 14 families (including three monocot families: Amaryllidaceae, Poaceae, and Liliaceae) Barow and Meister [18] showed the most significant factor determining whether or not a species underwent endopolyploidy was the particular life strategy adopted. It was observed to occur in species as a way to accelerate growth and was noted to be more frequent in annual and biennial herbs than perennials and absent in woody species. More recently, Barow and Jovtchev [19] reviewed the occurrence of endopolyploidy across angiosperms and listed 18 families (eight monocots and 10 eudicots) with predominantly endopolyploid species and ten families (three monocots and seven eudicots) with predominantly nonendopolyploid species.

2.4. GC Content of Genome. A number of papers have reported differences in nucleotide composition between monocot and eudicot genomes. These include differences in the %GC content at both the whole genome level and for individual genes. In both cases, the range of %GC values for monocots was wider compared with eudicots [21–23]. Nevertheless, many of these studies were based on analyses of just a few species in which all the monocot examples were taken from Poaceae. In more recent large-scale analyses, which extend to other monocot orders including Acorales, Asparagales, and Zingiberales, the picture is less clear [24–26]. Although species in Poales continue to show marked differences in their GC profiles compared with eudicots, analysis of the overall genomic %GC, the GC content of genes, and the distribution of GC content within coding sequences reveals that species belonging to some monocot orders are more similar to eudicots than Poales (e.g., *Acorus*; Acorales, *Asparagus*; Asparagales and *Allium*; Asparagales) whereas other species have GC profiles with characteristics shared by both eudicots and Poaceae (e.g., *Musa*; Zingiberales). A strong divide in genomic composition in terms of GC content and organisation does not therefore seem to exist between monocots and eudicots

3. Differences between the Genomes of Monocots and Other Angiosperms

Despite these overall similarities there are some genomic features that are distinctive in monocots, and these include the apparent greater flexibility in how DNA is organized into chromosomes and the amount of DNA comprising the genome.

3.1. Chromosome Packaging and Organization. In terms of chromosome packaging and organization, cytological investigations to date have suggested that the presence of holocentric chromosomes (i.e., those lacking a localized centromere) are more common in monocots than the rest of the angiosperms. Although the number of times they have arisen may be similar between these two groups (i.e., three families in each), the total number of species with holocentric chromosomes is greater in monocots. For example, they have been reported to be frequent in Cyperaceae which comprises c. 3,600 species [27–30], Juncaceae (comprising c. 325 species) [31], and the genus *Chionographis* (Melanthiaceae) (comprising c. seven species, [32]). In contrast, in the rest of the angiosperms they have so far only been noted in the nutmeg, *Myristica fragrans* (Myristicaceae) [33], c. 28 species of the parasitic *Cuscuta* subgenus *Cuscuta* (Convolvulaceae) [34, 35], and *Drosera* (c. 80 species) (Droseraceae) [36].

Similarly, available data suggest that the packaging of DNA into a bimodal karyotype organization (i.e., karyotypes comprising two distinct sizes of chromosomes) is more common in monocots (especially in Asparagales, Alismatales and Liliaceae, [37–39]) than the rest of the angiosperms where they have been reported in far fewer species (e.g., *Rhinanthus minor* (Orobanchaceae) [40] *Acantholepis orientalis* (Asteraceae) [41], *Onosma* (Boraginaceae) [42, 43], and some Australian *Drosera* [44].

Organization of the DNA at the telomeres of chromosomes also shows greater variability in monocots than in other angiosperms [45]. Whereas nearly all nonmonocot species analysed to date have been shown to contain typical *Arabidopsis*-like telomeric sequences at the ends of their chromosomes (i.e., $(TTTAGGG)_n$) (the exception being three genera of Solanaceae *Vestia*, *Cestrum* and *Sessea*) [46],

Table 1: Representation of available genome size data for angiosperms at different taxonomic levels using taxonomic circumscriptions given by Soltis et al. [2].

Taxonomic level	Number recognised	Number with genome size data available	Representation(%)
Families[1]			
Early diverging angiosperms	8	6	75
Eudicots	307	158	51
Monocots	78	64	82
Genera[2]			
Early diverging angiosperms	19	12	63
Eudicots	c. 10,400	795	8
Monocots	c. 2,800	534	19
Species[3]			
Early diverging angiosperms	268	24	9.0
Eudicots	220,000	3663	1.6
Monocots	c. 52,000	2527	4.9

[1] Based on APG III [4]. [2&3] Based on Mabberley [20].

in monocots, a large clade within Asparagales (comprising c. 6300 species) has replaced the *Arabidopsis*-type sequence with the human-type telomere sequence $(TTAGGG)_n$ [47–49] in the majority of species examined. Species of *Allium* were shown to be the exception even to this with no recognisable minisatellite so far identified [50].

3.2. Genome Size Diversity. Probably one of the most distinct differences is the diversity of genome sizes encountered in monocots compared with other angiosperms. Whereas several previous studies highlighted differences in the profile of genome sizes between monocots and dicots (e.g., [21, 51], both based on analyses of 2802 species), here the analysis is considerably extended to encompass the much larger and more representative genome size data set now available (see below) together with the more robust phylogenetic framework on which to analyze the data.

3.2.1. Data Available for Analysis. The Plant DNA C-values database [52] currently contains genome size data for 4427 angiosperms including 1885 monocot species. These values were combined with a further 1861 genome size estimates for species not already listed in the database but published in the literature to give a data set comprising 6288 species (including 2527 monocots). Table 1 shows the percentage representation for each of the major groups of angiosperms at different taxonomic levels.

4. Genome Size Diversity across Angiosperms

Across angiosperms, genome sizes range nearly 2000-fold from a 1C-value of just 0.063 pg in *Genlisea margaretae* (Lentibulariaceae) [53] to over 125 pg in tetraploid *Fritillaria assyriaca* (Liliaceae) [54]. This makes them one of the most variable groups of eukaryotes in terms of genome size. Nevertheless, a histogram showing the distribution of different genome sizes (Figure 1) reveals that most species have very small genomes, with a mode, median, and mean genome size of just 0.6, 2.6, and 6.2 pg, respectively (N.B. the 1C value corresponds to the DNA amount in the unreplicated gametic nucleus).

To examine how this diversity of genome size data is distributed within a phylogenetic context and to compare monocots with the rest of the angiosperms, data were superimposed onto the summary topology of angiosperms given by Soltis et al. [2] (Figure 2). The topology combines data from the three-gene, 567-taxon data set of Soltis et al. [55, 56] modified in light of more recent data arising from the analyses of nearly complete plastid genome data sets of Jansen et al. [57] and Moore et al. [58]. As Figure 2 shows, the large diversity of genome sizes is not spread evenly across all angiosperm groups. Thus although all clades contain species with small genomes, species with very large genomes occur in isolated clades within the monocots and eudicots.

There are clear differences between monocots and eudicots, and this is seen by comparing their genome size profiles (Figure 3). Not only is the maximum DNA amount of monocots (1C = 127.4 pg) nearly 40% bigger than the largest eudicot genome (1C = 79.3 pg) for which we have data, but also the mean and median values are significantly larger (Figures 3(a) and 3(b)). The differences are particularly apparent if we focus in on the upper end of the range (Figures 3(c) and 3(d)). In eudicots the largest genome sizes so far reported are found in two mistletoe species (1C = 79.3 pg in *Viscum cruciatum* and 1C = 76.0 pg in *Viscum album,* both with $2n = 20$) [59, 60]. These are, however, clearly outliers as they are nearly twice the size of the next largest eudicot genome in the genus *Hepatica* (*H. nobilis* var. *pubescens;* $2n = 4x = 28$; 1C = 44.6 pg) in Ranunculaceae [61]. As Figure 3(c) shows, even this is an outlier as 99.5% of all eudicots have genomes smaller than 25 pg. In contrast there are many more monocot species possessing large genomes with 10% having genomes bigger than 25 pg based on the current sample (Figure 3(d)).

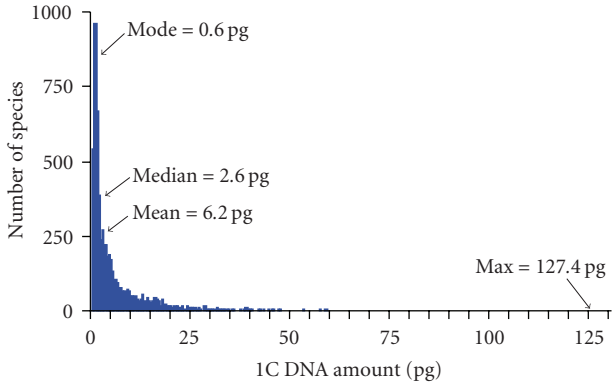

Figure 1: Histogram showing the distribution of DNA amounts in 6288 angiosperm species.

5. Genome Size Diversity within Monocots

Within monocots the 637-fold range of genome sizes is not distributed evenly across orders (Figure 4(a)); instead, distinct differences in the genome size range, mean, median, and modal values (Table 2) and profiles are apparent (Figure 4(b)). All orders have species with small genomes, whereas those with larger genomes (i.e., 1C > 25 pg) are phylogenetically restricted.

Across angiosperms as a whole there is no overall clear correlation between genome size and total chromosome number ($2n$), and chromosomes can vary in size without any change in DNA depending on the nutrient status of the plant [62–64]. Nevertheless, many studies have highlighted the potential for using chromosome data as proxies for genome size (e.g., [38, 65–68]). Thus in the survey of genome size diversity in monocot orders presented below, the data have been supplemented with the more comprehensive chromosome information that is available. Taken together, it is apparent that different monocot orders follow distinctive modes of genome size and chromosome evolution.

5.1. Acorales. This order comprises a single genus with two species, *Acorus gramineus* and *A. calamus*. Small genome sizes for both species have been reported with 1C = 0.4 pg for *A. gramineus* and 1C = 0.7 pg for *A. calamus*, although no chromosome counts were given [69]. Nevertheless since only diploid counts have been recorded for *A. gramineus* so far ($2n = 24$) whereas triploids ($2n = 36$) and tetraploids with $2n = 44$ and 48 have been noted for *A. calamus* with small chromosomes (c. 1-2 μm in *A. calamus* [70–72]), it is suggested that the larger genome size reported for *A. calamus* is probably from a tetraploid cytotype.

5.2. Alismatales. Genome size data are available for 106 species in 12 of the 13 families within this order [4] and range from 1C = 0.3 pg in two species of Araceae (*Spirodela polyrrhiza* with $2n = 80$ and *Pistia stratiotes* with $2n = 28$) to 1C = 24.1 pg in *Zamioculcas zamiifolia* (although no chromosome count was reported, previous ones have all been $2n = 34$) [73]. As in monocots as a whole (Figure 3(b)), the distribution of genome sizes in this order is skewed towards the smaller sizes (Figure 4(b)), with only two families (Alismataceae and Araceae) possessing genomes larger than 5 pg. Polyploidy has played a role in generating these larger genomes but the predominant mechanism has been through increases in chromosome size, with some of the largest chromosomes so far reported being found in species with relatively low chromosome numbers in Alismataceae, Hydrocharitaceae, and Araceae [73–77]. Indeed, the species with the highest chromosome number and a genome size estimate is *Lemna minor* (Araceae) with $2n = 126$ and yet its 1C-value is just 1.5 pg [78]. Similarly, the highest chromosome number so far reported in Alismatales is $2n = 12x = 168$ in *Arisaema heterophyllum* (Araceae) [79]; however, its chromosomes are small (c. 1 μm), and its genome size is thus unlikely to exceed 24 pg.

5.3. Petrosaviales. Currently there are no genome size estimates available for the two genera in Petrosaviaceae, *Petrosavia* (three species) and *Japanolirion* (one species). Nevertheless, karyotype information suggests that this small family is characterized by relatively small genomes. Tamura and Takahashi [80] reported *Petrosavia sakuraii* to have $2n = 60$ with chromosomes ranging in size from 1.0 to 3.6 μm and Satô [81] noted a bimodal karyotype of $2n = 24$ for *Japanolirion osense* comprising three long and nine short pairs of chromosomes ranging from 0.4 to 3.1 μm. Satô also noted that the karyotype of *J. osense* was similar to *Chionographis japonica* (Melanthiaceae), and since the genome size of *C. japonica* has been estimated to be 1C = 1.53 pg (J. Pellicer, pers. comm.), this suggests that the genome size for *J. osense* will be of similar magnitude.

5.4. Pandanales. With just eight genome size estimates (1C = 0.4–1.5 pg) in four of the five families, representation in Pandanales is poor. In addition, attempts to supplement this information with cytological data are hampered because obtaining counts in some families has been reported to be extremely difficult (e.g., Cyclanthaceae [82]). This is partly due to the small (i.e., <2 μm) and, in some cases, numerous chromosomes that characterise Pandanaceae ($2n = 30$ (*Freycinetia*) and 60 (*Pandanus*) [83, 84]), Cyclanthaceae ($2n = 18$–32), and Velloziaceae ($2n = 14$–48) [85]. Nevertheless, based on available karyotype data even smaller genomes may be found in this order as one of the smallest genomes so far reported is in *Xerophyta humilis* (Velloziaceae) with 1C = 0.54 pg and $2n = 48$. However, South American *Xerophyta* species with only slightly larger chromosomes (i.e., up to 2.5 μm) but with $2n = 14$ have been reported (e.g., *Xerophyta minima*) [85].

At the other end of the scale, larger genomes may be found in Stemonaceae in which chromosomes may reach 7 μm in some species, although the highest chromosome number so far reported is $2n = 24$ [86]. Triuridaceae may also contain large genomes as this family contains species with chromosomes up to 17 μm in *Sciaphila dolichostyla* [87] although, like Stemonaceae, chromosome numbers do not exceed $2n = 48$.

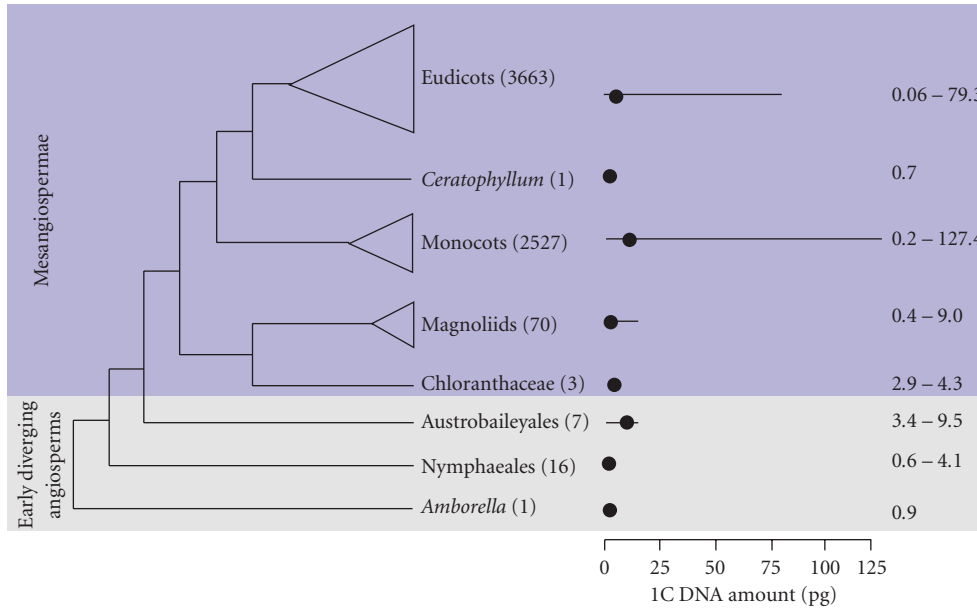

Figure 2: The phylogenetic distribution of genome sizes for 6288 angiosperm species. Summary topology based on Soltis et al. [2] is shown on the left while C-value data are given on the right. These show the mean (•) and range (represented as a line) of genome sizes encountered in each group. The number in brackets gives the number of species with genome size data.

5.5. Dioscoreales. This order, sister to Pandanales, contains three families (Dioscoreaceae, Burmanniaceae, and Nartheciaceae) and is poorly represented for genome size data. Estimates are available for just 14 species, 12 for *Dioscorea*, one for *Tacca* (both Dioscoreaceae), and one for *Narthecium* (Nartheciaceae). These data show a narrow range of genome sizes from 1C = 0.41 pg in *Narthecium ossifragum* to 6.75 pg in *Dioscorea elephantipes*. Although all families are characterized by possessing small to very small chromosomes (e.g., see [88–91]), high levels of polyploidy have been reported, particularly in Burmanniaceae and Dioscoreaceae in which chromosome counts of $2n = 136$ and c. 140, respectively, have been recorded [92–94]. Such karyotype information suggests that genomes larger than 6.8 pg may well be found as representation of genome size data improves. Nevertheless, since increases in ploidy are often accompanied by decreases in chromosome size (as noted in Nartheciaceae by Larsen [95] and by Sen in Burmanniaceae [89]), the upper limit of genome size in this order is unlikely to be very large.

5.6. Liliales. Circumscription of families and genera comprising Liliales has been considerably revised in recent years with ten families now recognized based on the combined analysis of five DNA regions and morphological characters [4, 96]. In contrast to other monocot orders, a histogram showing the distribution of genome sizes for 142 species from seven of these families is not strongly skewed to the left but is more evenly distributed (Figure 4(b)), and this is reflected in the highest mean 1C value of 39.26 pg for monocots (Table 2). It is here that the truly giant plant genomes are found with the record holders going to tetraploid *Fritillaria assyriaca* (Liliaceae, $2n = 48$, 1C = 127.4 pg) and *Trillium rhombifolium* (Melanthiaceae, $2n = 6x = 30$, 1C = 111.5 pg). However, very large genomes (i.e., 1C > 35 pg) [97] are not uncommon in genera belonging to subfamily Lilioideae of Liliaceae (e.g., *Lilium, Cardiocrinum, Notholirion, Tulipa,* and *Erythronium*) [38, 98, 99], tribe Parideae in Melanthiaceae (e.g., *Paris, Daiswa*), and *Alstroemeria* (Alstroemeriaceae). Although there are currently no genome size data for species in the saprophytic family Corsiaceae, probably sister to all remaining Liliales, very large genomes may also be encountered here given that the chromosomes were reported to be similar in size to those of *Pogonia* (Orchidaceae) [100], the genome size of which has recently been estimated to be 1C = 55.4 pg [101] (N.B. both species are reported to have $2n = 18$; see [100, 102]).

Cytologically, Liliales are as diverse as other monocot orders with a wide range of chromosome numbers ($2n = 10$–216) ploidy (up to 22x), bimodal karyotypes (e.g., *Alstroemeria, Luzuriaga, Rhipogonum, Smilax,* and many genera in subfamily Lilioideae, Liliaceae), and holocentric chromosomes (*Chionographis*, Melanthiaceae). However, it is perhaps notable that to date no species of Liliales have been reported with very small chromosomes (i.e., <1 μm) or genomes (i.e., <1.4 pg), as encountered in all other monocot orders. The smallest genome so far reported is in *Chionographis japonica* (Melanthiaceae, 1C = 1.53 pg, J. Pellicer, pers. comm.).

5.7. Asparagales. Around half of monocots are Asparagales (which comprise 14 families sensu [4]). The order includes five highly species-rich families (Orchidaceae, c. 25,000 species; Amaryllidaceae, c. 1,600 species; Asparagaceae, c. 2,500 species; Iridaceae, c. 1,900 species, Xanthorrhoeaceae, c. 850 species), with the remaining families containing between one and 36 species. Accompanying the species

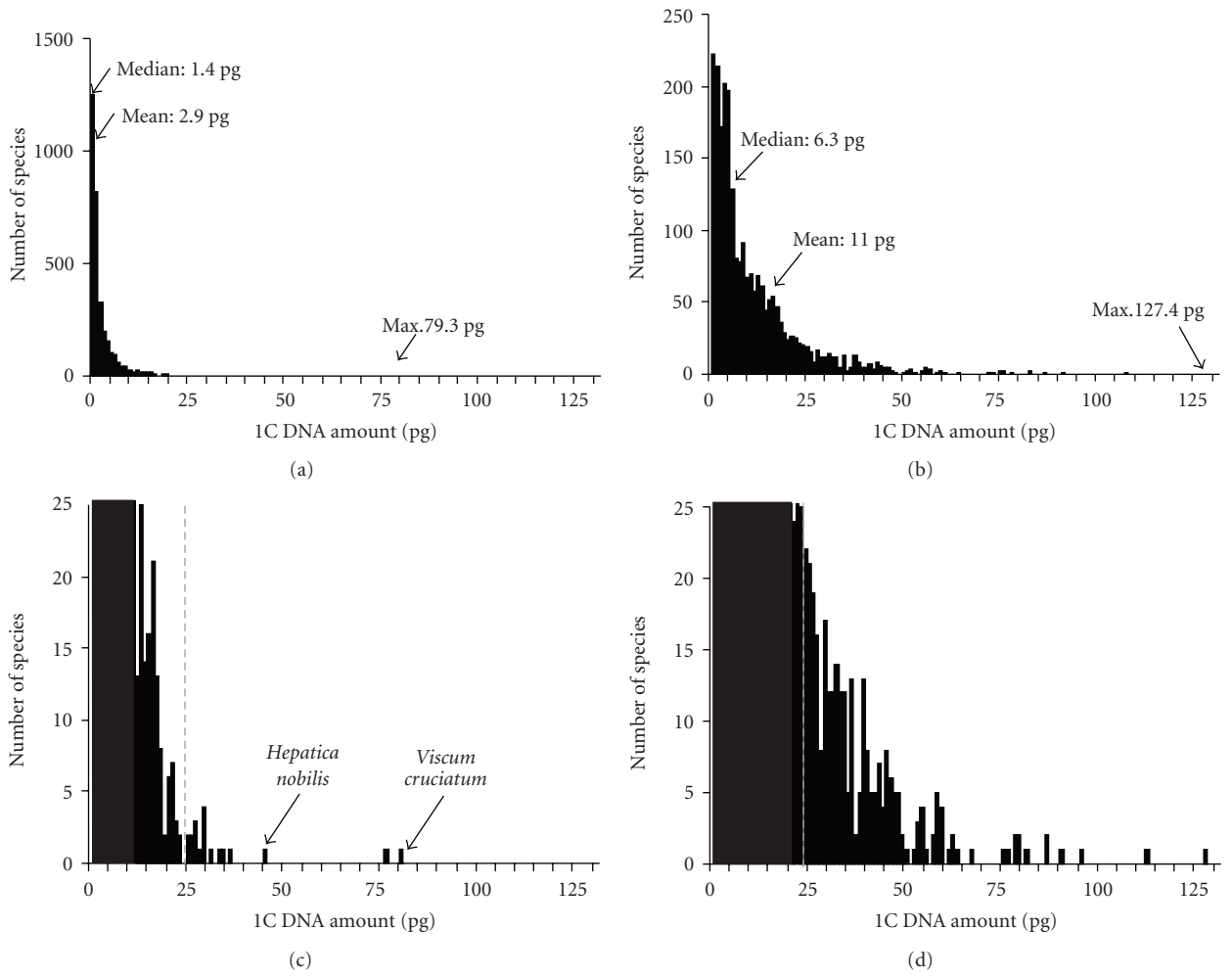

Figure 3: Histograms comparing the distribution of genome sizes in 3663 species of eudicot (a) and (c) and 2527 species of monocots (b) and (d). (a) and (b) show the full histograms for each group whereas (c) and (d) show the upper end of the range in greater detail. The dotted line corresponds to a 1C-value of 25 pg.

richness of the order is huge variation in chromosome number ($2n = 4$–228), karyotype structure (with bimodality being common in many genera), and modes of chromosome evolution [37].

From a genome size perspective, data are available for 1130 species in 12 of the 14 families and show that they too vary considerably (c. 250-fold from 1C = 0.33 pg in *Trichocentrum maduroi* (Orchidaceae) to 1C = 82.15 pg in hexaploid *Galanthus lagodechianus* (Amaryllidaceae), the largest range for any monocot order; Table 2). Nevertheless, the modal genome size is just 1C = 3.9 pg, and half of all species with data have genomes smaller than 11 pg, giving rise to the strongly skewed distribution of genome sizes (Figure 4(b)). Within the order it is clear that genome size diversity is restricted to the five species-rich families mentioned above (Figure 5), with Orchidaceae having the largest range for any family so far reported (168-fold, 1C = 0.33–55.4 pg) [101]. Genome sizes in the species-poor families do not exceed 1C = 8 pg. This is generally supported by chromosomal data as none of the smaller families is characterized by large chromosomes, and in Asteliaceae, where counts up to $2n = 210$ have been reported, the chromosomes are noted to be very small [103]. The only possible exception is Hypoxidaceae in which *Hypoxis obtusa* is reported to have $2n = 210$ [104]. Although there are currently no genome size data for any species of *Hypoxis*, a related species *Rhodohypoxis milloides* with $2n = 4x = 24$ has a genome size of 1.4 pg suggesting that genomes larger than 8 pg may occur in this family [105].

The largest chromosomes are found in Amaryllidaceae in *Haemanthus* (up to 24 μm in the predominantly diploid genus with $2n = 16$) and *Lycoris* (up to 28 μm in a genus where diploid chromosome numbers range from $2n = 12$ to 22 via Robertsonian translocations) [106–108].

5.8. Commelinids

5.8.1. Dasypogonaceae. This small family, comprising four genera (*Dasypogon, Calectasia, Kingia,* and *Baxteria*) and c. 8 species, is poorly known both cytologically and from a genome size perspective. Currently there is just a single

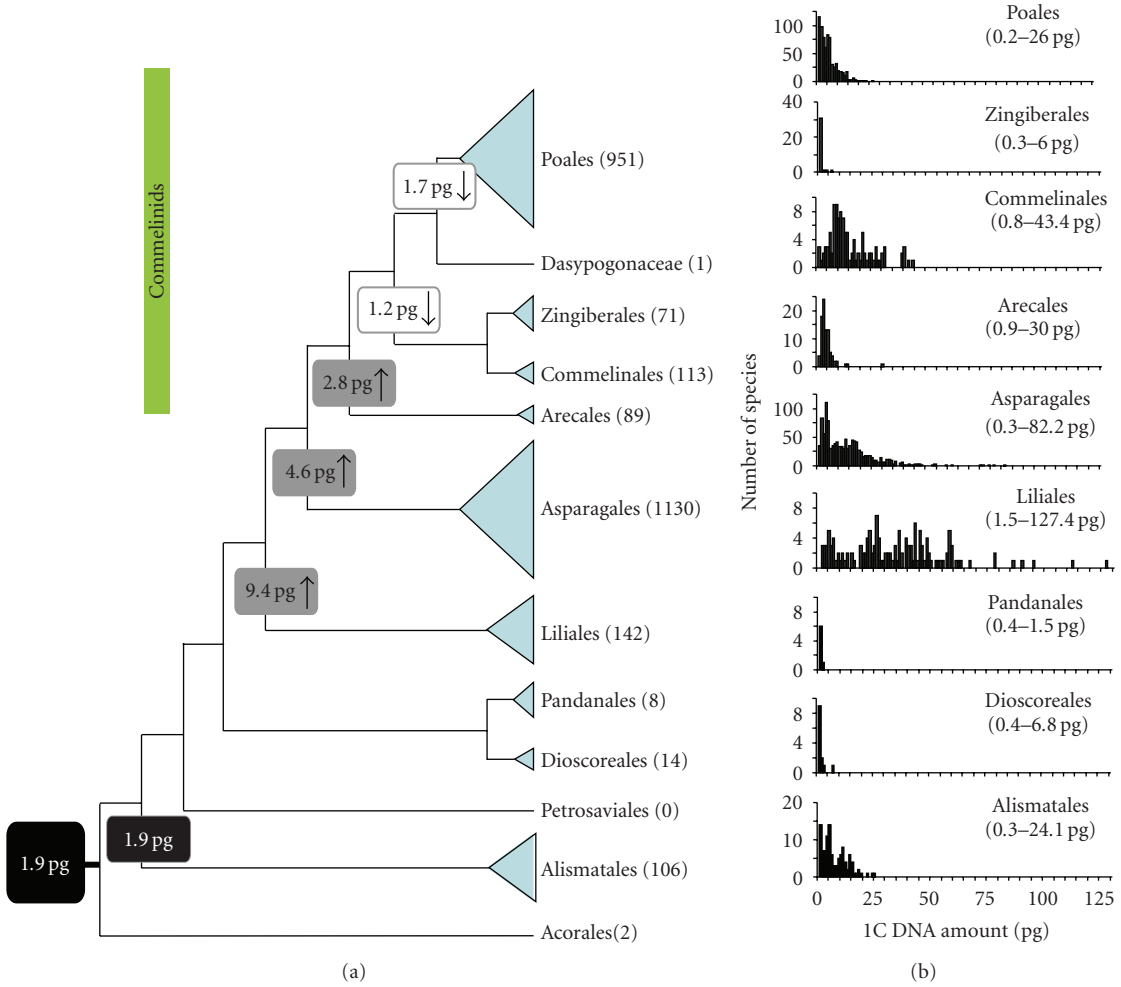

Figure 4: The phylogenetic distribution of genome sizes for 2527 species of monocots. (a) The summary topology of monocots (based on [3]) with the number in brackets corresponding to the number of species with genome size data. The size of the ancestral genome (1C-value) reconstructed at different nodes of the topology using BayesTraits is also given. Arrows indicate direction of genome size change relative to the ancestral genome size reconstructed at the base of all monocots (1C = 1.9 pg). (b) Histograms showing the distribution of genome sizes within each order, with the range of 1C-values given in brackets

genome size estimate for *Dasypogon hookeri* with 1C = 0.44 pg and $2n = 14$ [109].

5.8.2. Arecales. In the palm family Arecaceae (the only family of Arecales), genome size data are available for 89 species in 57 of the 183 recognized genera and representing all five subfamilies (Figure 6(a)) [110]. C-values range c. 33-fold from 0.9 pg in the diploid *Phoenix canariensis* (Coryphoideae) ($2n = 36$) [111] to 30.0 pg in the highly polyploid *Voanioala gerardii* (Arecoideae) with $2n$ = c. 600 [8]. The large C-value for *V. gerardii* is, however, clearly an outlier (Figure 4(b)) with the next largest genome size belonging to diploid *Pinanga subintegra* with 1C = 13.9 pg. This reflects cytological data showing that polyploidy is rare in palms with just four polyploid species reported to date, two tetraploids (*Arenga caudata,* $2n = 64$, and *Rhapis humilis,* $2n = 72$) and two rare, monotypic genera of high ploidy, c. 12x in *Jubaeopsis caffra* from South Africa ($2n$ = 160–200) and c. 38x in *Voanioala gerardii* from Madagascar [16]. The latter two genera belong to the same subtribe, Attaleinae, of tribe Cocoseae.

At the diploid level, genome sizes still range 13.9-fold, and this diversity contrasts with the narrow range of chromosome numbers reported across the c. 2,500 species (i.e., $2n = 26$, 28, 30, 32, 34, and 36). Röser [112] proposed that different chromosome numbers had evolved mainly through dysploidy due to the broadly similar DNA amounts in three related genera differing in chromosome number (*Livistona*, $2n = 36$; *Johannesteijsmannia*, $2n = 34$; *Licuala*, $2n = 28$). This is supported by an analysis of the larger data set available here. A comparison of the mean DNA amount for each chromosome number showed that they were not significantly different (data not shown). It is however clear that changes in genome size can occur with no alteration of chromosome number leading to related species having significantly different sized chromosomes (Figure 6(b)). The

Table 2: Genomic characteristics of the major monocot lineages

	Range of chromosome numbers ($2n$)	Occurrence of bimodal karyotypes	No. of species with C-values	Genome size data			
				Min. 1C-value (pg)	Max. 1C-value (pg)	Mean 1C-value(pg)	Range of 1C DNA amounts (max./min.)
Acorales	24–48	No	2	0.40	0.70	0.55	1.8
Alismatales	10–168	Common	106	0.30	24.05	7.16	80.2
Petrosaviales	24–60	Yes	0	—	—	—	—
Dioscoreales	12–140	Yes (*Thismia*, Burmanniaceae)	14	0.41	6.75	1.31	16.5
Pandanales	14–60	Yes but rare (only in Triuridaceae)	8	0.40	1.50	0.92	3.8
Asparagales	4–228	Common	1130	0.33	82.15	12.81	248.9
Liliales	10–216	Common	142	1.53	127.4	39.26	47.5
Dasypogonaceae	14-72	Yes in *Calectasia*	1	0.44	0.44	0.44	—
Arecales	26–c. 600	No	89	0.9	30.00	3.88	33.3
Commelinales	8–170	Yes but not common (only in some genera of Commelinaceae)	113	0.78	43.40	14.78	55.6
Zingiberales	8–105	No	71	0.30	6.00	1.06	20.0
Poales	4–266	Uncommon	951	0.20	26.00	4.81	130.0

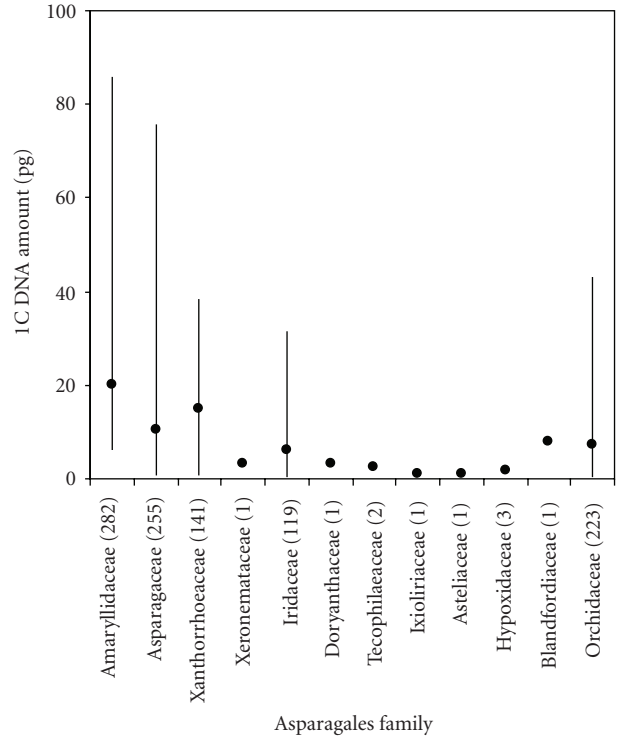

Figure 5: Range of 1C DNA amounts encountered in each of the 12 families of Asparagales for which genome size data are available. The number in brackets represents the number of species with genome size data. Familial circumscription follows APG III [4].

most dramatic example of this in palms is found in *Pinanga* where C-values range from 1C = 6.7 pg in *P. celebica* to 13.9 pg in *P. subintegra,* although all species have $2n = 32$ [110]. This is the largest range of genome sizes for any palm genus, and the possibility that it is linked to the diversity in reproductive evolution and speciation in *Pinanga* has been suggested by Loo et al. [113].

5.8.3. Zingiberales. Genome size-estimates are available for 71 species with at least one for each of the eight families comprising Zingiberales. The data show that this order is characterised by a narrow range of small genome sizes (1C = 0.3–6.0 pg). This reflects the more extensive cytological data indicating that the order is typified by karyotypes in which chromosomes are either all very small (i.e., <c. 2 μm; Marantaceae, Heliconiaceae) or small (i.e., c. 2–5 μm; Cannanaceae, Musaceae, Strelitziaceae, Costaceae), or in which the karyotypes contain a few larger chromosomes (6 or 7 μm; Lowiaceae, Zingiberaceae) as well as smaller ones. In addition, polyploidy is not widespread in the group as most species studied to date are cytologically diploid. It is only in a few genera of Zingiberaceae (e.g., *Cucumis, Hitchenia, Hedychium, Globba, Boesenbergia*) that polyploidy has played a significant evolutionary role, reaching 15-ploid in *Curcuma raktakonta*, with $2n = 105$, the highest chromosome number so far reported in Zingiberales [114, 115]. As in other monocot groups, however, due to the small size of the chromosomes in *C. raktakonta,* its genome is not the largest for Zingiberales (1C = 2.2 pg) [114]. Instead this is found

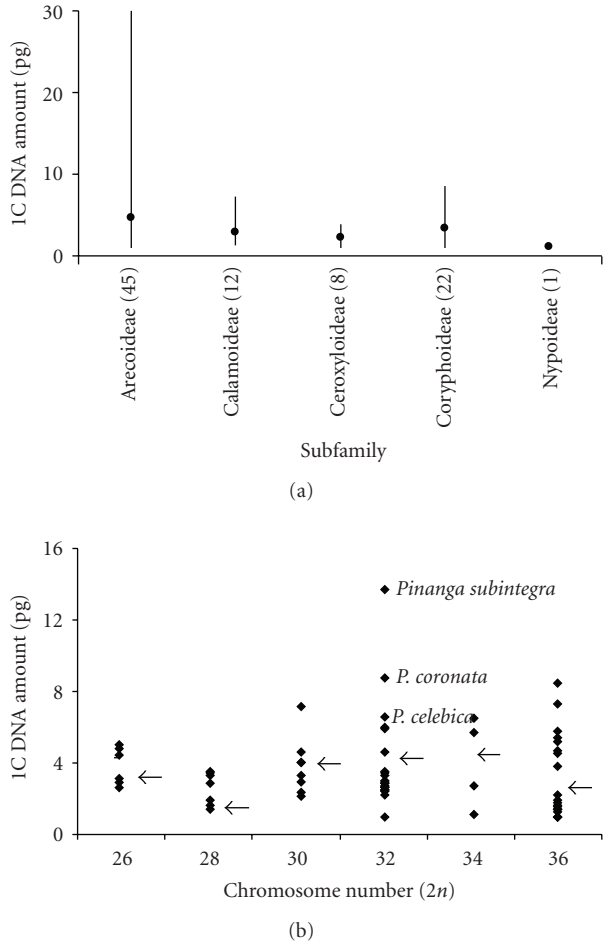

Figure 6: Genome sizes in palms (Arecaceae). (a) Range of DNA amounts in each subfamily (• = mean for subfamily). The number in brackets represents the number of species with genome size data. (b) Distribution of genome sizes within each chromosome number (arrows point to the mean for each data set).

in the diploid *Zingiber officinale* (1C = 6.0 pg, $2n = 22$) [116]. The smallest genomes in Zingiberales are found in diploid species of *Calathea* (Marantaceae) and *Heliconia* (Heliconiaceae) with 1C-values of 0.3-0.4 pg [105, 117]. Very small genomes are also found in two tetraploid species of *Maranta* (*M. arundinacea* $2n = 48$, 1C = 0.4 pg and *M. bicolor* $2n = 52$, 1C = 0.5 pg) leading to the possibility that even smaller genomes may be found in diploid *Maranta* species such as *M. arundinacea* var. *variegatum* with $2n = 18$ and small chromosomes (<2 μm) [118].

5.8.4. Commelinales. Within Commelinales, although genome size estimates are available for 113 species and range 56-fold, the data are highly unrepresentative with 108 values from Commelinaceae and the remaining five from Haemodoraceae (two species), Hanguanaceae (one species), and Pontederiaceae (two species) (there are currently no genome size estimates for Philydraceae, the last family in Commelinales). Genome sizes from the last three families are the lowest for the order ranging from just 0.8 pg in *Xiphidium caeruleum* ($2n = 38$; Haemodoraceae) [119] to 1.6 pg in *Hanguana malayana* ($2n$ = c. 170; Hanguanaceae) [109]. This narrow range reflects cytological data showing Haemodoraceae, Hanguanaceae, and Pontederiaceae to be characterised by possessing small to very small chromosomes. Nevertheless, polyploidy and dysploidy are also prevalent, particularly in Pontederiaceae in which chromosome numbers range $2n = 14$–80, and in Hanguanaceae with $2n = 90$–c. 170 [109, 120] suggesting that larger genome sizes within these families may be uncovered.

The most species-rich family by far is Commelinaceae with c. 650 species, and genome sizes here range 17-fold (1C = 2.6–43.4 pg). Even here, however, the data set is unrepresentative, being dominated by estimates from just three out of the c. 40 genera (i.e., *Tradescantia,* 52 species; *Gibasis*, 17 species; *Commelina,* 17 species). Nevertheless, given the extensive cytological data available for Commelinaceae (reviewed in [121–123]) the upper limit may not be extended considerably as the largest chromosomes so far reported belong to *Tradescantia virginiana* and its North and Central American allies [124], and the largest genome size estimate available is for tetraploid *T. virginiana* ($2n = 4x = 24$) with 1C = 43.4 pg [125]. Indeed the genomes that appear as outliers in Figure 4(b) (with 1C = 38.7–43.4 pg) are all tetraploid (where known) *Tradescantia* or *Callisia* species from N. America.

Nevertheless, it seems likely that Commelinaceae genomes smaller than 1C = 2.6 pg (for tetraploid *Commelina erecta,* $2n = 4x = 60$) will be uncovered as the smallest chromosomes so far reported are in *Stanfieldiella* with $2n = 22$, *Bufforestia* ($2n = 34$) and *Cartonema* with $2n = 24$ [121, 122, 126]. Not only are the chromosome numbers of these genera lower than *C. erecta,* but also the chromosomes are considerably smaller [121, 122, 127]. Very small genomes may also be found in *Pollia,* a genus noted to contain species with a low number ($2n = 10$) of very small chromosomes by Jones and Jopling [121].

5.8.5. Poales. Genome size-estimates are available for 951 out of an estimated 18,325 species in Poales (an order comprising 16 families) [4]. A summary of the range and distribution of genome sizes encountered in the twelve families with data is given in Table 3 and Figure 7.

Phylogenetically, within Poales there are some well-supported groups. Both molecular and morphological data suggest that Typhaceae and Bromeliaceae are probably sister taxa and form a clade sister to Rapateaceae and the remainder of Poales (see Figure 7) [3]. From a genome size and chromosomal perspective, data for two of these families show that they are characterised by small genomes comprising numerous very small to small chromosomes (Typhaceae $2n = 30$, 60, Bromeliaceae $2n$ = mostly 50 with occasional polyploids with $2n = 100$ and 150) [128–131]. For Rapateaceae there are no genome size data and only a few chromosome counts ($2n = 22$ and 52) [132] with no pictures. Thus insights into their genomes are currently lacking.

The remaining families are split into two large, well-supported clades; (i) the cyperid clade (comprising Xyridaceae, Eriocaulaceae, Mayacaceae, Thurniaceae, Juncaceae, and Cyperaceae) and (ii) the graminid clade containing

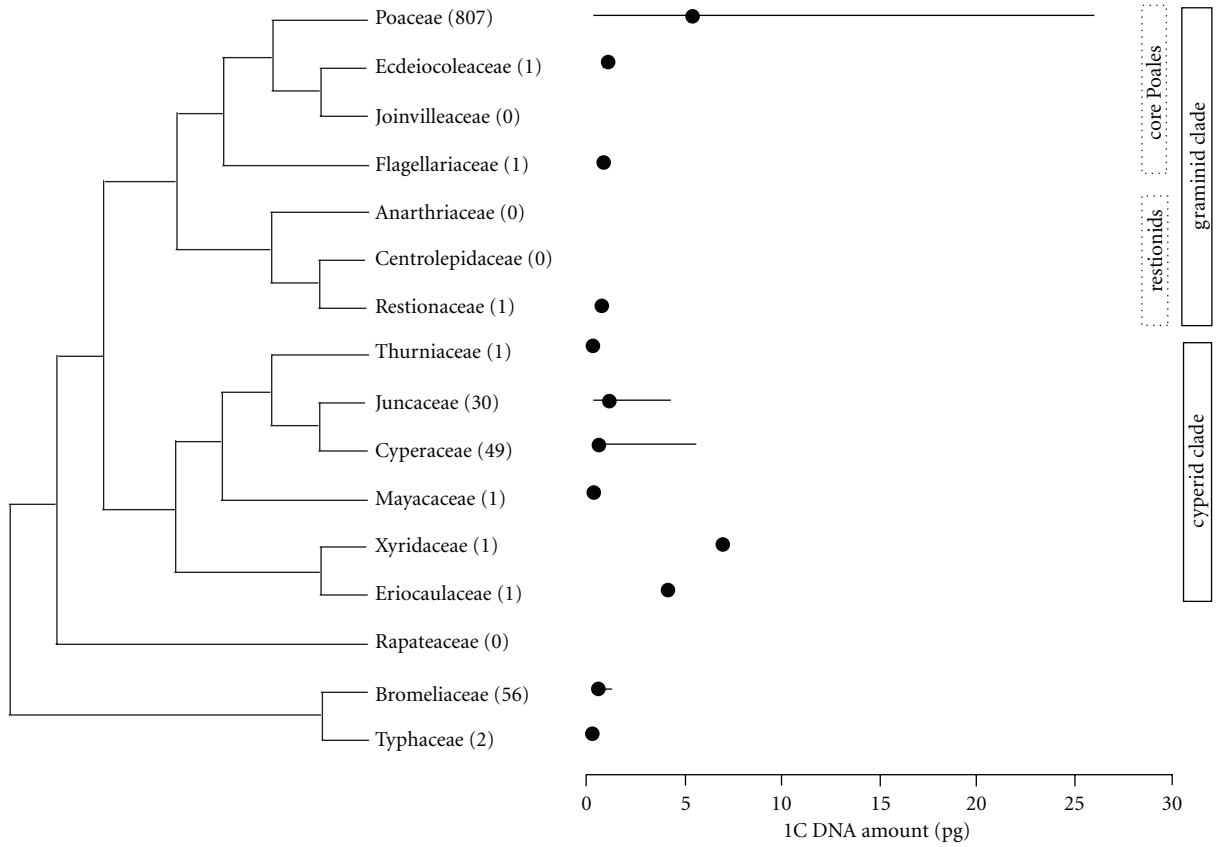

Figure 7: Phylogenetic distribution of genome sizes across Poales: summary topology of Poales predominantly based on Stevens [5] (left). The number in brackets gives the number of species with data. The mean (•) and range (represented as a line) of genome sizes for each family are shown in the middle. The major subdivisions of Poales are shown in blocks on the right.

the restionids (Anarthriaceae, Centrolepidaceae, and Restionaceae) and core Poales (Flagellariaceae, Joinvilleaceae, Ecdeiocoleaceae, and Poaceae).

The Cyperid Clade. Within the cyperid clade there is only one genome size estimate of 1C = 0.49 pg for Mayacaceae (*Mayaca* cf. *fluviatilis*; Smarda and Bureš, pers. comm.) and only very limited chromosome counts; thus inferences about their genomes are difficult. For the remaining families both Xyridaceae and Eriocaulaceae (two families often considered to be sisters) are characterised by small but highly variable chromosome numbers ($2n$ = 18–110) with polyploidy and dysploidy being important evolutionary mechanisms generating this diversity [133]. However, only two genome size-estimates are available (Table 3); so the full extent of genome size variation that accompanies chromosome diversity is currently unclear.

Based on molecular and morphological data, Juncaceae and Cyperaceae form a well-supported clade, most likely sister to Thurniaceae [3]. Genome size data are very sparse in Thurniaceae with currently just one genome size estimate (available for *Prionium serratum* of 1C = 0.33 pg; Smarda and Bureš pers. comm.) and no chromosome data. In contrast both Juncaceae and Cyperaceae have received considerable cytological attention because of the presence of holocentric chromosomes [27, 134]. Such studies have uncovered an extensive range in chromosome numbers (Cyperaceae $2n$ = 4 − c. 200; Juncaceae $2n$ = 16–130). Indeed, chromosome evolution is considered to be more

Table 3: Minimum (Min.), maximum (Max.), and mean 1C-values for the twelve families of Poales with genome size data.

Family	Number of C-value estimates	Min. 1C (pg)	Max. 1C (pg)	Mean 1C (pg)
Bromeliaceae	56	0.30	1.26	0.59
Cyperaceae	49	0.20	5.50	0.59
Ecdeiocoleaceae	1	0.99	0.99	0.99
Eriocaulaceae	1	4.19	4.19	4.19
Flagellariaceae	1	0.90	0.90	0.90
Juncaceae	30	0.30	4.30	1.00
Mayacaceae	1	0.49	0.49	0.49
Poaceae	807	0.30	26.00	5.52
Restionaceae	1	0.74	0.74	0.74
Thurniaceae	1	0.33	0.33	0.33
Typhaceae	2	0.30	0.50	0.40
Xyridaceae	1	7.01	7.01	7.01

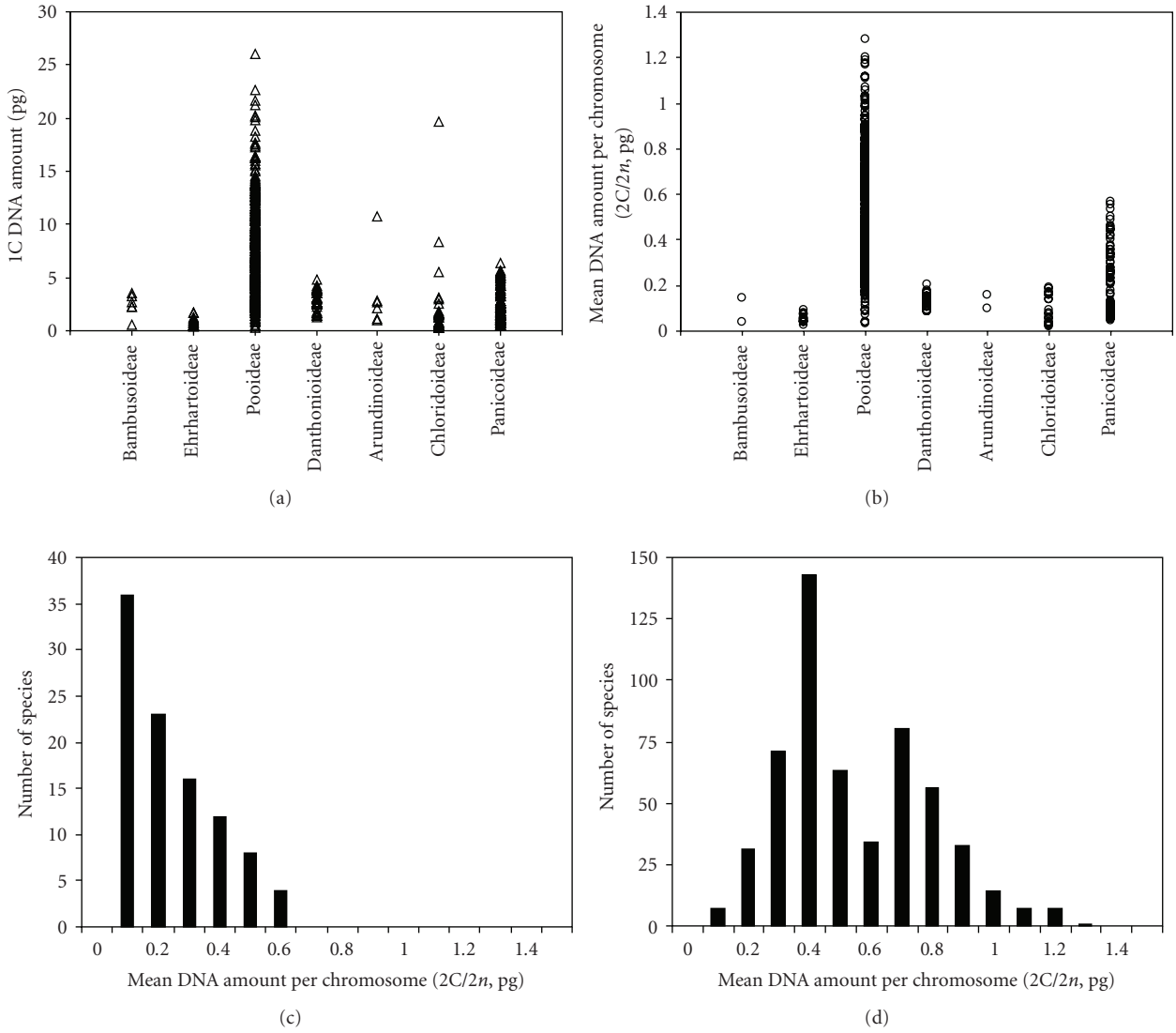

Figure 8: Range of 1C DNA amounts (a) and mean chromosome sizes (2C value/2n) (b) in each subfamily of Poaceae. Distribution of mean chromosomes sizes in Panicoideae (c) and Pooideae (d).

dynamic in *Carex* than in any other group of flowering plants with a series of chromosome numbers ranging from $n = 6$ to $n = 66$ [28, 135]. Across Cyperaceae and Juncaceae, polyploidy, agmatoploidy (increase in chromosome number through fragmentation of holocentric chromosomes), and symploidy (fusion of holocentric chromosomes) are considered to have been important in generating the diversity of chromosome numbers observed [27], with symploidy being so extensive in *Rhynchospora tenuis* (Cyperaceae) that its chromosome number has dropped to just $2n = 4$ [7].

Studies on genome size-evolution in taxa with holocentric chromosomes are more limited (e.g., [31, 136–138]) but available data show that the narrow ranges of genomes sizes encountered in the two families are similar (see Table 3, Figure 7). In general the average chromosome size (obtained by dividing the 2C value by the chromosome number) varied considerably across the range of chromosome numbers encountered, suggesting that chromosome evolution by symploidy and agmataploidy is often accompanied by considerable loss or addition of DNA [31, 139].

The Graminid Clade. Within graminids, the restionids form a well-supported clade that is diverse in chromosome number ($2n = 14$–104) and size (<1 μm in *Lepidobolus* (Restionaceae) to over 10 μm in *Anarthria* (Anarthriaceae)) [140, 141]. Currently, there is just one genome size estimate for *Rhodocoma gigantia* (Restionaceae) (1C = 0.7 pg) with no chromosome data; so how typical it is for this clade is unclear [109].

Within core Poales the three families related to Poaceae are poorly characterised both cytologically and from a genome size perspective. Available data suggest that they may possess small genomes as chromosome counts of $2n = 36$ (Joinvilleaceae), 38 (Flagellariaceae), and 42 and 64–66 (Ecdeiocoleaceae) have been reported, and the chromosomes are noted to be small [141–143]. The two

genome size estimates available support this (i.e., *Flagellaria guineensis*, 1C = 0.9 pg, $2n = 38$ (Flagellariaceae) [109] and *Ecdeiocolea monostachya* 1C = 1.0 pg, $2n$ = c. 38 (Ecdeiocoleaceae)[144]).

In contrast, Poaceae, one of the most species-rich angiosperm families (c. 10,000 species), has the greatest number of genome size estimates for any family in the monocots with values for 807 species and extensive chromosome data with numbers ranging from $2n = 4$ to 266. Given this large amount of data together with extensive genomic and phylogenetic information available for grasses there have been numerous studies on the evolution of grass genomes and their sizes [145–147]. Indeed, many of the insights into the molecular basis and evolutionary dynamics of genome size-variation in angiosperms as a whole have been gained through the study of grass genomes [148–150]. These have revealed the rapid and dynamic nature of genome size-evolution in grasses [146, 151], the mechanisms involved in generating genome size-diversity [152], and the contribution that transposons and in particular retrotransposons have made to genome size-differences [153–156] and highlighted contrasting patterns of genome size and chromosome diversification in the grass subfamilies [146, 157].

Analysis of the 807 genome estimates reveals that subfamilies are characterized by different ranges of genome sizes. All subfamilies contain species with small genomes, whereas species with genomes greater than 1C = 14 pg are restricted largely to Pooideae and one species of Chloridoideae (*Bouteloua gracilis* 1C = 19.7 pg) (Figure 8(a)). Some of this variation can be attributed to polyploidy as species with genomes larger than 1C = 14 pg are all polyploid (4x–c. 38x), and although a count was not made for *B. gracilis*, previous records show it to range $2n = 28$–77 (4x–11x) with small chromosomes (c. 0.5–2 μm) [158]. Indeed, by plotting the distribution of mean chromosome sizes for each subfamily (by dividing 2C values by chromosome number) to remove the effect of polyploidy, the largest chromosomes are found in Pooideae and Panicoideae with all the other subfamilies being characterized by much smaller chromosomes (Figure 8(b)). Once again, species with high chromosome numbers in the data set have some of the smallest chromosomes and relatively small genomes (e.g., *Spartina anglica* $2n = 122$, 1C = 5.5 pg; *Cenchrus caliculatus* $2n = 102$, 1C = 5.6 pg [159]). The largest chromosomes are found in diploid species of *Secale* and *Psathyrostachys* with $2n = 14$ (both Pooideae).

The average chromosome sizes for the two largest subfamilies (Pooideae and Panicoideae) are distinct. Although most species in Panicoideae are characterized by relatively small chromosomes (with a modal DNA amount per chromosome of 0.1 pg (Figure 8(c)), there are two modal peaks in chromosome size at 0.4 and 0.7 pg for Pooideae (Figure 8(d)) suggesting that the evolutionary processes driving chromosome and genome size-evolution are different in these subfamilies. Even within subfamilies differences in the rates of genome change are apparent. Genomic comparisons between four grass genomes suggested that the rate of genome evolution in *Aegilops tauschii* (Pooideae) was substantially higher than *Brachypodium distachyon* (Pooideae), *Sorghum bicolor* (Panicoideae), and Oryza sativa (Ehrhartoideae) [160].

6. Evolution of Genome Size Diversity within Monocots

From the above discussion it is clear that the different orders of monocots have undergone very different patterns of both genome size and chromosome evolution, giving rise to the genomic diversity observed. In seeking to understand how such diversity in genome size evolved over the c. 110–120 million years since monocots first appeared in the fossil record [161], ideally one would aim to obtain genome size estimates from key fossil taxa. However, although various approaches have been suggested for using fossil epidermal or guard cells as proxies for genome size [162, 163], the poor and patchy fossil record for monocots has precluded such an approach so far.

An alternative line of attack is to use statistical modelling to reconstruct genome size evolution. However, although there have been several studies that have used these approaches to reconstruct the size of the ancestral genomes across the angiosperm tree, including monocots (e.g., [164, 165]), many have analysed genome size as a discrete character requiring the data to be partitioned into size classes. Since genome size varies continuously, a biologically more meaningful approach is to analyse it as a continuous character, and there are now numerous studies that have used such approaches to analyse genome size within particular plant genera and families (e.g., [98, 166, 167]). Recently we have been extending the application of these approaches to examine genome size-evolution across monocots as a whole, not only to reconstruct ancestral genome sizes at different nodes of the monocot tree but also to compare rates of genome size evolution to see whether the different genomic profiles observed in the monocot groups (Figure 4(b)) are reflected in differences in the mode and tempo of genome size-evolution. The full details of the methods and approaches used are outlined in Beaulieu et al. (in prep.), and a summary of the findings is presented here.

The two statistical modeling programs used for analysis were BayesTraits [168–170] and Brownie [171]. BayesTraits applies a generalized least square approach to model genome size evolution. It provides insights into the mode and tempo of genome size evolution and also reconstructs ancestral genome sizes at different nodes within the phylogenetic tree. In contrast, Brownie uses maximum likelihood to analyse rates of genome size evolution across a phylogenetic tree and it can be applied to test for substantial differences in the rate of genome size-evolution between monocot clades.

Using these approaches, the following picture of genome size-evolution in monocots is emerging.

6.1. The Ancestral Genome Size of Monocots. The ancestral genome size of all monocots was reconstructed as 1.85 pg (Figure 4(a)), similar to previous studies using MacClade in which the ancestral genome size was reconstructed as being "very small" (i.e., 1C $\leq$ 1.4 pg [164]. Within monocots,

our analysis showed that there was a general tendency for increases in the ancestral genome size such as at the base of Arecales (1C = 2.8 pg) and Asparagales (1C = 4.6 pg) and a large increase at the base of Liliales (1C = 9.4 pg). In contrast, decreases were observed within commelinids in the branch leading to Commelinales and Zingiberales (1C = 1.2 pg) and a slight decrease at the base of Poales (1C = 1.7 pg).

To what extent the predicted increases in ancestral genome size in Asparagales, Arecales, and Liliales reflect signatures of an ancient whole genome duplication near the base of all monocots is unclear although support for such an event is increasing based on the expanding sequence data being generated in key monocot species (see [14]). Alternatively, the larger ancestral genome sizes in these groups may reflect whole genome duplication events at or near the base of each clade. Already, an analysis of the *Acorus* genome has uncovered evidence of at least one round of polyploidy [13]. Whether multiple polyploid events occurred at or near the base of Liliales to contribute to an ancestral genome size (1C = 9.4 pg) more than five times that of monocots as a whole (1C = 1.85 pg) remains to be seen but requires sequence data from key species of Liliales that sadly are not currently available.

The predicted decreases in ancestral genome size along the branches leading to Poales and Zingiberales + Commelinales suggest that the origin of these clades may not have been accompanied by a whole genome duplication event, and this is supported by available sequence data that have failed to find evidence of polyploidy in these phylogenetic positions [172]. Nevertheless, polyploidy within these clades has clearly taken place especially within Poaceae based on both chromosomal and DNA sequence analyses. Cytologically 80% of all Poaceae are estimated to be polyploid [173], and an inferred whole genome duplication event 50–70 million years ago in Poaceae has been proposed (e.g., [174, 175]), close to the origin of Poaceae (c. 89 mya) [176]. Within Zingiberales sequence data have provided evidence for a whole genome duplication c. 60 mya in Musaceae but not in Zingiberaceae [26].

Nevertheless, it is clear that all groups of monocots analysed contain species with genomes smaller than the reconstructed ancestral genome size which highlights the propensity for genome size to decrease as well as increase.

6.2. Mode and Tempo of Genome Size Evolution in Monocots. The mode of genome size evolution was shown to be that of "scaled gradualism" meaning that genome size has evolved in a gradual rather than punctuated manner over time but with more changes in the shorter branches than the longer branches of the phylogenetic tree used for analysis. This suggests that the rate of genome size evolution slows down on the longer branches. Genome size evolution was also shown to be "slow" rather than "accelerated" suggesting that most diversity in genome size encountered in monocots was established early. This is consistent with studies in *Orobanche* (Orobanchaceae) [166] and Brassicaceae [177], which pointed to a slow tempo of genome size evolution implying that most of the diversity of genome sizes encountered in these two eudicot groups evolved early on in their diversification. In contrast, an accelerated tempo of genome size evolution was recently reported in a similar, but more focused study of genome size evolution in Liliaceae by Leitch et al. [98]. It is, however, too early to say to what extent these patterns reflect differences between monocots and eudicots and further studies are clearly needed.

Using Brownie the results showed that despite different genome size profiles of the clades analysed (Figure 4(b)) there was no evidence to suggest that clades with bigger genomes (Liliales) were evolving more rapidly than other clades. Instead all the major monocot clades were shown to be evolving at nearly the same rates with the exception of Asparagales and Poales, which were shown to be evolving at significantly higher rates than other monocot clades. Whether the elevated rates occur across the whole order or are restricted to specific families and genera within Asparagales and Poales needs to be investigated further. In recent studies of chromosome and sequence evolution in Poaceae, an accelerated rate of structural genome evolution was shown to be restricted to species in Triticeae with larger genomes when compared with relatives in other tribes with smaller-genomes [151]. Indeed, there may be a link between the activity of transposable elements, their rate of turnover, and genome size-evolution since species with larger genomes have been observed to have more interchromosomal duplications than species with smaller genomes [178]. Additional work is needed to extend these studies beyond Poaceae.

7. Future Directions

The picture emerging from current large-scale comparative sequence analyses of plants is that genomes of Poaceae are different from other monocot lineages that appear more eudicot-like [25, 26]. However, out of the nine plant genomes "completely" sequenced so far, the only monocots all belong to Poaceae (i.e., *Oryza sativa, Sorghum bicolor, Zea mays,* and *Brachypodium distachyon*). The anticipated release of complete genome sequences for some other grass genomes (e.g., *Triticum aestivum*) will no doubt add to the power of comparative analysis, but the need for species in other families and clades is clearly apparent if one is to really get to grips with the diversity of monocot genomes and how distinct they really are from other angiosperms.

8. Note Added in Proof

Following the acceptance of this paper the authors were made aware of a paper by Zonneveld [179] which is also published in this special issue. Zonneveld presents the first genome size estimate for the triploid hybrid *Trillium* x *hagae* ($2n = 30$) with a 1C DNA amount of 132.5 pg. As this is larger than *Fritillaria assyriaca* (1C = 127.4 pg), the range of genome sizes encountered in angiosperms and land plants as a whole has now increased to 2056-fold, while the range for monocots has increased to 665-fold. Zonneveld also reports new 1C-value estimates for the eudicot *Viscum album* (102.9 pg) and *V. cruciatum* (87.9) pg. Both values are higher than

those reported previously for the same species. Using these values also extends the range of genome sizes encountered in eudicots to c. 1633-fold.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 989152, 8 pages
doi:10.1155/2010/989152

Research Article

On the Tempo of Genome Size Evolution in Angiosperms

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Received 4 January 2010; Accepted 17 April 2010

Academic Editor: Jan Suda

Broadly sampled phylogenies have uncovered extreme deviations from a molecular clock with the rates of molecular substitution varying dramatically within/among lineages. While growth form, a proxy for life history, is strongly correlated with molecular rate heterogeneity, its influence on trait evolution has yet to be examined. Here, we explore genome size evolution in relation to growth form by combining recent advances in large-scale phylogeny construction with model-based phylogenetic comparative methods. We construct phylogenies for *Monocotyledonae* (monocots) and Fabaceae (legumes), including all species with genome size information, and assess whether rates of genome size evolution depend on growth form. We found that the rates of genome size evolution for woody lineages were consistently an order of magnitude slower than those of herbaceous lineages. Our findings also suggest that growth form constrains genome size evolution, not through consequences associated with the phenotype, but instead through the influence of life history attributes on the tempo of evolution. Consequences associated with life history now extend to genomic evolution and may shed light on the frequently observed threshold effect of genome size variation on higher phenotypic traits.

1. Introduction

The concept of a "molecular clock" predicts that nucleotide substitution rates should scale linearly with time and therefore be equal among lineages. However, rarely do datasets conform to a molecular clock (e.g., [1]) and broadly sampled phylogenies have clearly documented dramatic lineage-specific molecular rate heterogeneity across the Tree of Life (e.g., [2–6]). Life history, or more specifically, generation time, is a strong correlate of among/within lineage rate heterogeneity in both animals and plants [6–8]. In plants, molecular rates are consistently more variable and typically higher in herbaceous species when compared to "woody" (i.e., trees/shrubs) species. Generation time may play a role in this pattern as herbaceous species typically have shorter generation times than woody species, and hence a greater capacity to accumulate nucleotide substitutions per unit time. Implicit in these results is a renewed appreciation for the link between microevolutionary process and macroevolutionary pattern [9].

The consistent pattern of life history influences on rates of molecular evolution across several loci [5, 6, 10] implies this pattern may manifest at the whole genome-level—the first phenotypic scale above molecules. The size of any given genome is determined by rates of DNA accumulation (e.g., retrotransposition and polyploidy) and deletions (e.g., via unequal crossing over and illegitimate recombination). The rate of genome size evolution is therefore set by the interplay between selection and drift promoting and eliminating these mutational changes [11–13]. Indeed, several phylogenetic studies have revealed increases and decreases in genome size [14–17].

Extant angiosperms exhibit a growth form dependent distribution in genome size. Woody angiosperms are characterized by small genome sizes with lower overall variance compared to herbaceous species [18, 19]. This asymmetry in genome size variance among growth forms has been interpreted as an indication of large increases in DNA content negatively impacting woody species [19, 20]. However, when viewed in the context of microevolutionary processes,

the growth form dependent distribution of genome size could also be explained in part by consequences associated with life history. For example, woody angiosperms take many years to reach reproductive maturity [21]. For genome size, this may allow fewer opportunities for insertion/deletions to occur per unit time. Therefore, in terms of generation time, the smaller and lower variance in genome size exhibited by woody species need not be explained only by functional constraints on the phenotype [19, 22].

Here, we test for growth-form dependent rates of genome size evolution between woody and herbaceous lineages. Specifically, we test whether woody species exhibit slower rates of genome size evolution than related herbaceous species. To explore genome size evolution in relation to growth form, we combine recent advances in large-scale phylogeny construction [23] with model-based phylogenetic comparative methods [24]. We focus our analyses on two major branches of the angiosperms that are well represented in the Plant DNA C-value database [25]: the *Monocotyledonae* (monocots; [26]) and the Fabaceae (*Leguminosae* or legumes). The monocots are a large clade of mainly herbaceous angiosperms that also contain a few clades of predominately woody species, including the palms (Arecales; [27]). The legumes are the third largest family of angiosperms and exhibit a wide range of growth habits throughout the clade. It is worth noting that unlike the woody legumes, "woody" monocots do not produce true "wood". In this context, however, we generally define the tree/shrub or "woody" category as simply large plants with long generation times, for example, [6].

2. Methods and Materials

2.1. Genome Size Data. The amount of DNA in the unreplicated gametic nucleus (i.e., pollen or egg) is referred to as the 1C DNA amount or holoploid genome size, regardless of ploidy level [28]. However, since many angiosperms undergo polyploidy, the monoploid genome size, or 1Cx value, is also often reported and analyzed. The monoploid genome size represents the amount of DNA in the unreplicated monoploid chromosome set and is calculated by dividing the 2C DNA amount by ploidy. Because rates of evolution can be inflated due to polyploidy, we compare and contrast evolutionary rates between the two measures (see below). We compiled genome size estimates for legumes and monocot species where both the 1C amount and the ploidy level were known. Data from the Plant DNA C-values database [25] were combined with additional genome size estimates not yet listed in the database but published in the literature, resulting in an initial list of 1659 and 565 monocot and legume species, respectively, to search GenBank (see below).

2.2. Mega-Phylogeny Construction. We constructed a *mega-phylogeny* of legumes and monocots using the procedures described in [23]. The *mega-phylogeny* method applies orthology tests, sequence saturation analyses, and multiple profile-to-profile alignment methodology to user-specified gene regions. Sequence saturation is detected by calculating the median absolute deviation (MAD) assessed on the one-dimensional Euclidean distance between the raw and Jukes-Cantor corrected pair-wise sequence distances. For a given gene region, if the most inclusive grouping of these sequences is saturated (MAD > 0.01) then the group is broken up into less inclusive groups using the next level in the NCBI (National Center for Biotechnology Information) taxonomic hierarchy. After every sequence has been placed in an alignment, the individual alignments are then "profile aligned" into a larger alignment. Profile-to-profile alignment combines separate alignments, while preserving the structural elements that are highly conserved between them [29, 30]. We employed a guide tree based on the phylogeny of the NCBI taxonomy to carry out profile alignments.

For the monocots, we specified *atp*B, *mat*K, *ndh*F, *rbc*L, *rps*16, *trn*L-F, and ITS as our gene regions of interest. For the legumes we specified *mat*K, *psb*A-*trn*H, *rbc*L, *trn*L-F, ITS, and ETS. However, instead of compiling all possible monocot and legume taxa for a given gene region, we limited our GenBank search to only return sequences for taxa represented in our genome size dataset. The mega-phylogeny matrix construction pipeline was carried out in Python (Ver. 2.5) with the BioPython (Ver. 1.48) module using the BioSQL (Ver. 1.0.1) database schema. Each phylogeny was inferred from the resulting matrix using RAxML (Ver. 7.0.4; [31]), partitioning each gene region and applying a GTRMIX model of rate substitution. For monocots, the maximum likelihood tree was rooted with Acorales (*sensu* [32]) and the legumes were rooted with the tribe Cercideae (*sensu* [33]). In both cases, due to synonymy and errors in Genbank, the trees were further pruned to match our genome size data sets (for a complete list see supplementary materials).

2.3. Time Calibrating the Mega-Phylogeny. We time-calibrated the legume mega-phylogeny using the nonparametric rate smoothing method (NPRS; [34]) with the Powell algorithm in r8s (Ver. 1.71; [35]). The NPRS analysis was restarted three times with different starting values to ensure convergence to a global optimum. We selectively assigned five age constraints from age estimates inferred by Lavin et al. [36]. These included the Umtzia crown group (54.0 million years ago, Mya), the Hologalegina crown (50.6 Mya), the *Vigna-Phaseolus* split (8.0 Mya), and one assigned to crown Fabaceae (59.0 Mya). We also assigned a constraint within the dalbergioid clade that corresponded to a node in our tree (49.1 Mya).

For the monocots, we selectively assigned eight age constraints using the mean absolute age estimates from Smith et al. [37]. Six age constraints corresponded to the crown age estimates for major clades of monocots (Asparagales, 99.8 Mya; Arecales, 70.9 Mya; Poales, 74.8 Mya; Zingiberales, 88.5 Mya; Commelinales, 76.8 Mya), two corresponded to deep divergences (Liliales + Asparagales, 121.3 Mya; crown Commelinids, 114.9 Mya), and one was assigned to crown monocots (163.5 Mya). We initially used the same procedure to date the monocot tree as above, but the nonparametric rate smoothing analysis did not run to completion. To deal with this problem, we reduced the dataset to 200 tips and reran the NPRS analysis to completion. We obtained the estimated ages for all nodes in the reduced dataset and placed

them in the full dataset. We then used the nonparametric dating method PATHD8 [38] to infer ages for the remaining uncalibrated nodes. PATHD8 uses mean path lengths from the node to tips and deals with substitution rate variation by smoothing rates locally.

2.4. Comparative Analyses. To test for differences in the rate of genome size evolution (1C and 1Cx DNA content) among woody and herbaceous lineages, we compared the fit of single- and two-rate models of Brownian motion evolution. Any phenotypic trait found to accumulate evolutionary change in proportion to time is best described by Brownian motion [39]. The time-independent parameter, σ^2, or the variance of phenotypic evolution, describes the rate at which this process proceeds. The single-rate model assumes that all analyzed branches accumulate evolutionary changes in genome size at the same rate, σ^2, while the multiple-rate model assigns a separate rate to each lineage that differs in a particular discrete character state (e.g., $\sigma^2_{\text{woody}}, \sigma^2_{\text{herb}}$). We carried out the single- versus two-rate model comparisons using the "noncensored" approach in BROWNIE (Ver. 2.1; [24]). Because the "noncensored" approach assumes the discrete character state of internal branches are known, we used a procedure implemented in BROWNIE that estimates the likeliest growth form state (e.g., woody or herbaceous) across all branches in a given tree based on character codings at the tips. Evaluating the best-fit model between the single- and two- rate models was based on the sample size corrected Akaike Information Criterion (AICc; [40]). The "best" fit model was chosen based on a slightly modified $\triangle$AICc. Because we are only comparing two models, we always calculated $\triangle$AICc as AICc obtained from the single rate model minus the AICc from the two-rate model. A $\triangle$AICc of <2 was taken as evidence for the single-rate model, whereas a $\triangle$AICc >2 indicated considerable evidence for the two-rate model.

We also tested for mean differences in genome size among extant woody and herbaceous species in both our monocot and legume datasets. However, many types of evolutionary processes could have produced the observed trait differences, including Brownian motion. Therefore, we assessed genome size differences among growth form and compared the results of a conventional ANOVA to a null distribution based on ANOVA results obtained from simulations of Brownian motion evolution [41]. This was used to test whether significant species differences between growth forms were larger than would be expected given a random model of Brownian motion evolution. We used the R [42] package GEIGER [43] to generate 1000 Monte Carlo simulations using our input tree topology and time-calibrated branch lengths. We compared the observed *F*-statistic calculated using an ordinary ANOVA to a null distribution of *F*-statistics obtained from the Monte Carlo simulations to test for significance. If the observed *F*-statistic was greater than 95% of the null distribution, then trait differences were greater than expected based on a model of Brownian motion evolution. We carried out this test within each clade separately, using both 1C and 1Cx DNA content.

We $\log_{10}$ transformed the genome size data prior to all analyses to ensure the data minimally conformed to Brownian motion evolution [23, 44]. Under a simple Brownian motion model of evolution (as we employ throughout), a given trait should have an equal probability of increasing or decreasing in the same magnitude given its current state. However, this assumption is inherently violated when traits, such as genome size, are constrained to be non-zero. For example, given a genome size of 0.25 pg, an increase or decrease of 0.50 pg is not likely to occur in equal probability. Rather, in this case, change would be better expressed as a proportion, where the probability of an increase or decrease of say, 50%, is likely to occur regardless of the initial genome size at speciation. Thus, it is generally acknowledged that genome size evolution may be better represented as proportional change through an *a priori* $\log_{10}$ transformation [23, 44].

3. Results

3.1. Mega-Phylogeny. Our final matrices for the *Monocotyledonae* (monocots) and Fabaceae (legumes) consisted of 495 and 250 species, respectively. The combined matrix for the legumes comprised 60 woody species from 20 genera and 190 herbaceous species from 21 genera. The woody species were mostly confined to the clades corresponding to the Cercideae, Mimosoideae, and Caesalpinioideae, with additional occurrences found within the Papilionoideae. For monocots, the matrix comprised 213 genera belonging to 9 of the 10 orders of monocots recognized by the Angiosperm Phylogeny Group [27]. Slow growing, tall and/or "woody" genera have been described in several different monocot families, including Arecaceae, (e.g., *Cordyline*, *Dasylirion*, *Dracaena*, *Nolina*), Bromeliaceae (e.g., *Puya*), Dasypogonaceae (*Dasypogon*, *Kingia*), Pandanaceae (*Pandanus*), Strelitziaceae (e.g., *Ravenala*), Velloziaceae (*Vellozia*), Xanthorrhoeaceae (e.g., *Aloe* and *Xanthorrhoea),* and the woody bamboo genera in the tribe Bambuseae of Poaceae (e.g., *Phyllostachys*, *Sasa, Semiarundinaria*). However, due to the absence of genome size and/or sequence data for many of these genera the effect of growth form analyses were restricted to comparisons between (*i*) *Dasypogon* (Dasypogonaceae; 1 species), the "woody" palms (Arecaceae; 34 species), and the "woody" *Aloe* (Xanthorrhoeaceae; 5 species), (*ii*) the remaining species which were classified as herbaceous (452 species).

The combined matrix for the monocots contained 10,922 sites and 74.5% gaps or missing sequence, while the legume matrix had a total length of 8221 sites that contained 80.4% gaps or missing sequence. In both cases, the majority of the sequence data came from ITS (Table 1). Additionally, the degree of saturation varied among gene regions, ranging from profiling broad clades (e.g., *rbc*L) to profiling mostly tribes and genera (e.g., ITS; Table 1). Interestingly, of the all genes sampled, only *rbc*L did not require some degree of profile alignment (Table 1). It is worth noting that the degree of saturation was not related to whether or not the gene was protein coding. For example, in both the legume and monocot data set, the noncoding *trn*L-F regions required as much profile aligning as the coding *mat*K (Table 1).

Table 1: Gene regions specified in the mega-phylogeny construction of *Monocotyledonae* (monocots) and Fabaceae (legumes). The median absolute deviation (MAD) was used to assess sequence saturation and to parse sequences into separate files based on NCBI taxonomy and brought together again using NCBI-based guide tree and profile-to-profile alignment methodology (see Methods and Materials).

Phylogeny	Gene region	Description	*N*	MAD	Profiles
Monocotyledonae	*atp*B	Atp synthase beta chain	128	0.006	none
Monocotyledonae	ITS	Internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2	596	***0.108***	mostly to tribe and genus
Monocotyledonae	*mat*K	Maturase K	310	***0.068***	mostly to family
Monocotyledonae	*ndh*F	NADH-plastoquinone oxidoreductase	203	***0.021***	mostly to order
Monocotyledonae	*rbc*L	Ribulose bisphosphate carboxylase	270	0.005	none
Monocotyledonae	*rps*16	Ribosomal protein S16 intron	88	***0.014***	mostly to order
Monocotyledonae	*trn*L-*trn*F	*trn*L-*trn*F intergenic spacer	472	***0.033***	mostly to family
Fabaceae	ETS	External transcribed spacer and 18S ribosomal RNA	18	***0.064***	mostly to tribe and genus
Fabaceae	ITS	Internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2	293	***0.031***	mostly to tribe and genus
Fabaceae	*mat*K	Maturase K	119	***0.014***	mostly to tribe and genus
Fabaceae	*psb*A-*trn*H	*psb*A-*trn*H intergenic spacer	31	0.005	none
Fabaceae	*rbc*L	Ribulose bisphosphate carboxylase	66	0.001	none
Fabaceae	*trn*L-*trn*F	*trn*L-*trn*F intergenic spacer	81	***0.040***	mostly to tribe

MAD scores in bold italics indicate the gene region was saturated across the most inclusive taxonomic-level and broken up into profiles of various taxonomic levels.
N indicates the number of sequences in GenBank returned according to our input search list; however, due to synonymy and errors in GenBank the final tree was pruned to exactly match our genome size data set.

Table 2: Parameter estimates from comparisons of single- versus two-rate models of Brownian motion (BM) and applied to both 1C DNA and 1Cx DNA content separately.

	1C DNA content				1Cx DNA content			
	Single-rate	Two-rate			Single-rate	Two-rate		
Clade	$\sigma^2(\text{My}^{-1})$	$\sigma^2_{\text{woody}}(\text{My}^{-1})$	$\sigma^2_{\text{herb}}(\text{My}^{-1})$	ΔAICc	$\sigma^2(\text{My}^{-1})$	$\sigma^2_{\text{woody}}(\text{My}^{-1})$	$\sigma^2_{\text{herb}}(\text{My}^{-1})$	ΔAICc
Monocotyledonae	0.0132	0.0018	0.0142	44.8	0.0055	0.0018	0.0058	17.7
Fabaceae	0.0444	0.0043	0.0552	85.4	0.0338	0.0041	0.0417	74.0

△AICc is calculated as the AICc obtained from the single rate model minus the AICc obtained from the two-rate model, where a △AICc < 2 was taken as evidence for the single-rate model, whereas a △AICc > 2 indicated strong evidence for the two-rate model.

3.2. Rates of Genome Size Evolution. In both the monocots and legumes, we found that the genome size data were best fit by a two-rate model of Brownian motion evolution, which inferred a separate rate for woody and herbaceous lineages (Table 2). For legumes, the two-rate model applied to the 1C DNA content was strongly supported (ΔAICc = 85.4) and woody lineages were inferred to accumulate changes in genome size an order of magnitude slower than related herbaceous lineages. Even when testing 1Cx DNA content, the disparity in rates between woody and herbaceous legumes remained (Table 2). In monocots, the two-rate model was also strongly favored (ΔAICc = 44.8) with accumulated changes in 1C DNA content occurring at a rate that was also an order of magnitude slower than related herbaceous lineages. Although a significant difference in rates was still detected (△AICc = 17.7), the discrepancy in inferred rates was somewhat reduced between growth forms when testing 1Cx DNA content (Table 2).

Across monocots, there were no significant differences in genome size among woody and herbaceous species ($F_{1,493} = 0.533, P = .904$). For the legumes, mean genome size was significantly smaller in woody species than herbaceous species ($F_{1,248} = 27.5, P < .001$). However, the phylogenetically informed ANOVA suggested that the mean values between woody and herbaceous species of legumes were significantly different, but no more different than would be expected under a model of gradual Brownian motion ($P = .253$). In other words, the observed mean differences among growth form could have arisen by chance alone.

4. Discussion

Our analyses demonstrated that the tempo of genome size evolution is strongly influenced by growth form. In both monocots and legumes, the best fitting model of evolution for genome size inferred a separate rate for each growth form,

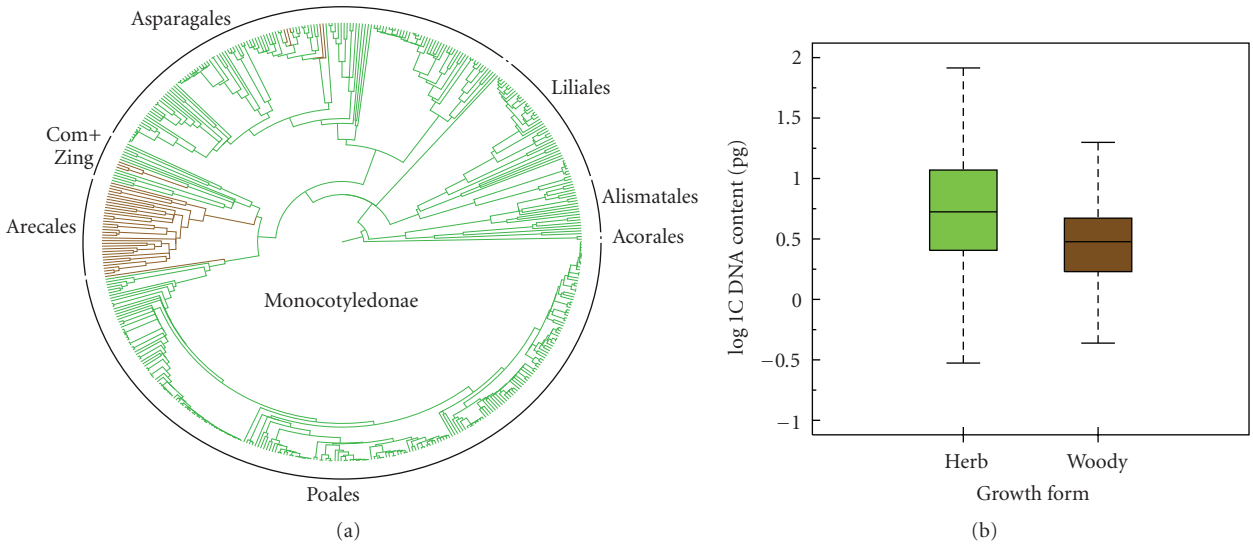

Figure 1: (a) Time-calibrated phylogeny of Monocotyledonae (monocots; [26]). Phylogeny is taken from a maximum likelihood analysis of 495 species based on combined analysis *atp*B, ITS, *mat*K, *ndh*F, *rbc*L, *rps*16, and *trn*L-F. The major clades of monocots are labeled, and estimates of the likeliest growth form state (woody = brown; herbaceous = green) across all branches in the tree. Com+Zing represents the combined clade of Commelinales and Zingiberales. (b) The distributions of 1C DNA content among growth form, we detect no significant differences among herbaceous (green) and woody (brown) monocots for both 1C DNA and 1Cx DNA content (not shown). The boxplot represents the median (central line), 1st and 3rd quartiles (gray box), and outliers.

with woody lineages accumulating changes in genome size at rates that were consistently an order of magnitude slower than related herbaceous lineages (Table 2). The pattern was consistent across not only two very distinct clades of angiosperms, but also two separate measures of genome size (1C and 1Cx DNA content; Table 2). Therefore, our results suggest that life history alone can impose constraints to the evolution of genome size. These constraints likely reflect the influence of generation time with the longer generation times that characterize woody species [21, 45] providing fewer opportunities for changes in genome sizes to occur per unit time (e.g., [46]).

Plants, unlike animals, do not sequester a germ line early in development, which has the potential for somatic mutations to accumulate throughout growth, particularly for plants with longer generation times. Indeed, there is evidence for greater somatic mutations in longer-lived species compared to annuals on a per generation basis [47–50]. Thus, if an increased number of somatic mutations also involve changes in genome size, then this would complicate any generation time explanation for the observed slower rate of genome size evolution in presumably longer-lived woody species. However, extensive intraindividual and intraspecific variation is not commonly observed for genome size [51–53] suggesting that although there is a potential for a greater number of somatic mutations in longer-lived species to contribute to genome size differences, this may not be a significant factor. The observed excess of new radial cell files in the vascular cambium of trees has been suggested to be one important mechanism for removing somatic mutations from the meristematic population [45, 54]. Likewise, various plant life cycle characteristics (e.g., pollen tube competition, interovule selection within the same ovary, selective seed/fruit abortion, etc.) have the potential to purge defective genotypes arising from both somatic and gametic mutations without markedly reducing reproductive capacity [47, 55]. Such characteristics may contribute towards explaining the observed reduction in accumulated mutations per unit *time* in woody species despite the potential for more mutations to accumulate on a per generation basis (e.g., [6]).

Additional life history correlates such as effective population size may also play a role, though they are less clearly associated with the observed disparity in rate. Angiosperm trees are reported to have large effective population sizes [45], which would make selection more efficient at removing deleterious mutations and excess DNA [13]. Stronger selection in woody species would be consistent with the suggestion that large increases in DNA content negatively affect woody growth and physiology [19, 20]. However, we found no significant phylogenetic differences in genome size between woody and herbaceous species in either the monocot or legume data sets (Figures 1 and 2), which would be expected if small genome sizes were a requirement for woody species [19]. Moreover, there was no consistent pattern of woody species having smaller genome size in genera consisting of both woody and herbaceous species. For example, within the primarily herbaceous genus *Medicago*, the only woody representative, *M. arborescens*, has a genome size that is nearly twice that of most other species in our dataset. This was also true within the monocot genus *Aloe* (Xanthorrhoeaceae) and is a general observation from

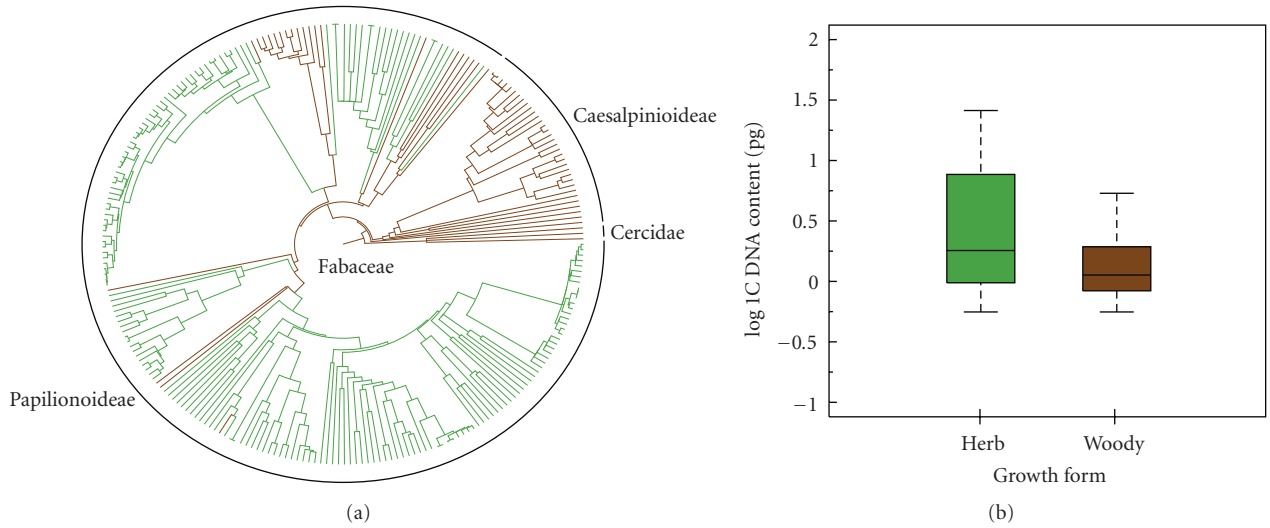

Figure 2: (a) Time-calibrated phylogeny of Fabaceae (legumes). Phylogeny is taken form a maximum likelihood analysis of 253 species based on a combined analysis of ETS, ITS, *mat*K, *psb*A-*trn*H, *rbc*L, and *trn*L-F. The major clades of legumes are labeled and estimates of the likeliest growth form state (woody = brown; herbaceous = green) across all branches in the tree. (b) The distributions of 1C DNA content among growth form, where we find significant differences among herbaceous (green) and woody (brown) legumes for both 1C DNA and 1Cx DNA content (not shown), but no more different than could arise by chance (see Methods and Materials). The boxplot represents the median (central line), 1st and 3rd quartiles (gray box), and outliers.

angiosperm genera not included in this study (see [18]). In addition, it is clear that not all woody plants possess small genome sizes, as the completely woody *Acrogymnospermae* (a clade containing the four major lineages of extant "gymnosperms"; [26]) are characterized by much larger genomes that are 12 times the modal value of angiosperms [56]. Nonetheless, the influence of selection and generation time may not be mutually exclusive, but assessing a potential asymmetry in selection due to growth form will require developing models of phenotypic evolution that allow decoupling of the strength of selection (e.g., Ornstein-Uhlenbeck model; [57, 58]) across discrete character states.

Small genome sizes are consistently associated with a large range of phenotypic variation that decreases with increasing genome size. This pattern has been documented for a suite of traits, including climate tolerance [59], leaf mass per unit area (LMA; [60]), maximum height [61], and seed mass [62]. For example, very large genome sizes do not produce small-sized seeds and species with small genome sizes exhibit a range of seed sizes [62]. While genome size may set the minimum seed mass due to size constraints at the cellular level (e.g., large genomes are not contained within small cells; [19]), it remains unclear why the largest seeds are not associated with large genomes. Perhaps, the observed upper constraint does not relate to genome size at all, but instead reflects the constraint imposed on genome size evolution by generation time. Trees and shrubs produce large seeds in comparison to herbaceous species [63, 64], suggesting that the preponderance of seed mass variation at smaller genome sizes may simply reflect a diversity of growth form. Because "woodiness" confers a marked reduction in the rate of genome size evolution, the decreasing phenotypic variation with increasing genome size may simply be a function of insufficient time having elapsed for woody angiosperm to evolve large genome sizes.

Further analyses should focus on large-scale comparisons of growth form dependent rates of genome size evolution in order to uncover a generality. In addition, more focused studies on specific life forms such as succulents, parasites, geophytes may also help to resolve and refine the interplay and influence of growth form on rates of genome size evolution. Such approaches require increased sampling efforts of genome size estimates for species across a broad range of taxonomic groups. Nevertheless, our results of monocots and legumes suggest that, in addition to molecular substitution rates [2, 5, 6], growth form can also influence the tempo of genome size evolution. Therefore, given the consistency across two scales–molecules and genomes–a logical next step is to examine higher phenotypic traits in relation to growth form. Only through combining trait databases (e.g., Glopnet [65]; SID [66], etc.) with the construction of broadly sampled phylogenies (e.g., [23]) will interesting life history trends continue to be uncovered.

Supplementary Materials

Tables 1 and 2 in the supplementary material list all species and associated growth form designation included in the combined mega-phylogeny matrix of monocots and legumes; see tables 1 and 2 in the supplementary material available online at doi: 10.1155/2010/989152.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 382732, 8 pages
doi:10.1155/2010/382732

Review Article

Recent Insights into Mechanisms of Genome Size Change in Plants

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Received 4 February 2010; Accepted 19 March 2010

Academic Editor: Ilia Judith Leitch

Genome sizes vary considerably across all eukaryotes and even among closely related species. The genesis and evolutionary dynamics of that variation have generated considerable interest, as have the patterns of variation themselves. Here we review recent developments in our understanding of genome size evolution in plants, drawing attention to the higher order processes that can influence the mechanisms generating changing genome size.

1. Introduction

It has long been known that tremendous variation in DNA content exists, even within closely related species, and that organismal complexity is poorly correlated with genome size. In plants, genome size ranges from 63 Mbp in *Genlisea margaretae* to 124,852 Mbp in *Fritillaria assyriaca* [1], a 2000-fold difference. This diversity of genome size has generated considerable interest in the nature of sequence variation among genomes, the mechanisms that operate to add and/or remove DNA, and the suite of internal and external evolutionary forces that collectively shape or control the molecular drivers. Key insights into each of these arenas have emerged in the recent years from the explosion of genomic sequence data. Here we provide a synopsis of this burgeoning field, focusing on the recent developments that have improved our understanding of the processes that underlie genome size change in plants.

2. Heterogeneity in Genome Size Fluctuations

After the initial puzzling observation that greater perceived complexity does not equate to a larger genome [2], it was quickly realized that the non-protein-encoding fraction of the genome comprised sequence types that actually correlated with genome size [3]. Repetitive elements, it was learned, and primarily LTR-retrotransposons in the case of plant genomes, could achieve surprisingly high copy numbers, by themselves accounting for half or more of the genome for species having "large" genomes [4]. Many subsequent studies have demonstrated that the fraction and composition of the genome occupied by these sequences reflects, mechanistically, the antagonistic effects of insertion, due primarily to transposable element (TE) proliferation, and deletion, primarily mediated by unequal intrastrand homologous recombination (i.e., recombination between directly repeated sequences, such as the LTRs of single or adjacent retrotransposons) and illegitimate recombination (i.e., *RecA* independent recombination capable of deleting sequence intervening regions of microhomology) [5–7]. To generate the extraordinary range in extant angiosperm genome sizes, it has been reasoned, the magnitude of these mechanisms must also vary among species.

That this is true has now been confirmed in multiple studies involving taxa distributed widely among angiosperms. Most notably, it has become apparent that differential proliferation of TEs explains the majority of genome size differences among species. In the wild rice species *Oryza australiensis*, for example, amplification of three TEs accounts for a 2-fold increase in genome size [8]. Similarly, evidence from *Gossypium* (cotton) [9] indicates that the majority of the threefold range in diploid genome

sizes may be accounted for by amplification of the *Gorge3 gypsy* element in the larger genomes (but see below).

One important dimension of the foregoing, and other studies, is the realization that TE proliferation is not a constant feature of plant genomes nor of any specific lineage, but that instead it is an episodic or saltational process throughout the angiosperms. Bursts of transposition have been inferred from TE dating analyses in many species [7, 8, 10, 11]. These and other studies also have revealed that different types of TEs (e.g., *copia* or *gypsy* LTR retrotransposons) or different subfamilies of a single type (e.g., *copia* LTR retrotransposons) may episodically proliferate at different times. The result is that lineages experience periodic quantum gains in genome size that are likely controlled by myriad factors (e.g., epigenetics, recombination rate, etc.), which vary by element type/family and, presumably, in response to genomic and environmental factors (e.g., hybridization or environmental stress).

In addition to heterogeneity in the amount of historical TE proliferation, deletional mechanisms also have been demonstrated to vary in importance among species. The first such survey of the relative importance of unequal intrastrand homologous recombination (UR) versus illegitimate recombination (IR) was conducted in *Arabidopsis thaliana*, where it was concluded that IR has had a larger impact than UR, removing more than fivefold DNA [12]. In rice, however, unequal homologous recombination has been more efficient at purging extraneous DNA (3.3 Mbp for UR versus 2.8 Mbp for IR) [13]. Since those two initial studies, the relative effectiveness of UR versus IR has been evaluated for different species [14–17] and the subject of their effectiveness relative to one another and relative to TE proliferation has been a topic of debate [5, 7]. That is to say, since IR often results in substantially smaller deletions than UR, the relative effectiveness of IR versus UR has been questioned, as has the ability of either to reverse, or even slow, genome size growth in the face of massive transposable element proliferation, both when considered individually or together. While it is clear UR has the ability to more rapidly remove large amounts of DNA, IR has a broader scope of action (i.e., it is not reliant upon sequence homologies, such as LTRs, to operate); therefore, the relative impact of each mechanism will vary as the number of potential sites for UR diminishes. New evidence (discussed below) also elucidates the effect genomic properties can have on both deletional mechanisms, which further underscores the impact that additional data will have in increasing our understanding of these mechanisms.

The picture that has emerged from an increased understanding of TE proliferation as well as deletional processes is one which surmises that extant genome sizes reflect the often oppositional processes of genomic expansion and contraction. Thus, for example, recently inserted TE DNA often is rapidly removed, potentially leading to rapid genomic turnover [18, 19] and even within species variation [20–22]. A comprehensive study of LTR-retrotransposons in rice showed that in addition to several bursts of transposition experienced during the last 5 million years, the rice genome also experienced a flurry of deletions, ultimately leading to removal of over half of the inserted LTR-retrotransposon DNA [11]. Similarly, Hawkins et al. demonstrated lineage-specific, differential removal of the TE most responsible for the threefold variation in genome size among *Gossypium* diploids, that is, the *gypsy*-like *Gorge3* element [23]. By phylogenetically partitioning *Gorge3* elements into time points representing: (1) pre-*Gossypium* amplification, (2) *Gossypium-specific* amplification, and (3) lineage-specific amplification, and utilizing a novel modeling approach, the authors were able to reconstruct the ancestral copy number for *Gorge3* and infer gains and losses for each lineage. A key conclusion is that the smaller genomes are not only *not* gaining *Gorge3* as quickly as the larger genomes, but they are also more effective in *removing* elements, and at a rate that actually exceeds the rate of gain.

This demonstration that genomic contraction via TE removal can actually exceed the rush toward genomic obesity [24] implied by bursts of TE proliferation is mirrored in an additional study in cotton using a phylogenetically informed approach to polarize small indels. Indels in two genomic regions were catalogued and characterized for five genomes among species whose phylogenetic relationships were well-established, thus providing the opportunity to interpret small indels as losses or gains of DNA sequence [16]. Differences in the *rates* of sequence gain and loss were demonstrated among terminal branches and between ancestor and descendent, demonstrating that temporal heterogeneity characterizes multiple mechanisms of genome size evolution. Overall, the trend for the diploid genomes, both extant and ancestral, was toward growth, albeit slowly in some cases; however, the polyploid experienced growth of one subgenome (D_T) and contraction of the other (A_T), resulting in a net loss for the polyploid genome. While many deletions contributed to overall contraction, the majority of sequence loss was attributable to the removal of a single *gypsy* element, once again underscoring the potential for rapid removal via UR.

Finally, it has become evident that insertion and deletion operate heterogeneously with respect to genomic location. This is clear from studies using from FISH [25, 26], sequencing of genomic regions [17, 27–31], and whole genome sequencing projects [32–35]. This unevenness is pronounced for the LTR-retrotransposon *gypsy* superfamily, whose members often experience a significant bias toward residing in genomic locations that are considered more heterochromatic in nature and most often are associated with pericentromeric or centromeric regions [32, 33, 35–38]. Other locational biases, however, have also been noted (e.g., euchromatic regions for copia LTR-retrotransposons and UTR/exonic regions for SINEs in maize [34, 35], gene-rich regions for maize *Mutator* and Helitron elements, [35, 39, 40], and introns for LINEs in maize and soybean [29, 41]). These observed biases likely reflect myriad factors, including but not limited to insertional preferences, disruptive potential for insertion in a given region, local rates of recombination (discussed below), and lineage-specific effects. The influence of genomic location on deletional mechanisms has been evaluated less; however, as UR acts largely on LTR-retrotransposons and both UR and IR are

recombination based, it is not hard to imagine the relevance of genomic location to these processes.

3. Epigenetics and Genome Size

While the principal mechanisms responsible for genome size expansion and contraction appear to be relatively clear, the factors that stimulate or control each mechanism remain enigmatic. Because of the heterogeneity in the operation of insertional and deletional mechanisms with respect to genomic region, lineage, and time, it stands to reason that this heterogeneity reflects multiple interacting external environmental forces as well as intrinsic genomic properties. One that has garnered much attention in recent years is epigenetic regulation of transposable elements.

Epigenetic regulation of transposable elements is considered to be the first line of defense against uncontrolled TE proliferation. Methylation and heterochromatization of TEs as a means to limit proliferation is not a recent idea, with observations in the Mutator system of maize representing some of the earlier research into epigenetic regulation of TEs [42–46]. More recently, the pathways by which TEs are silenced have been illuminated, specifically the dependence upon RNAi to silence transcription and remodel TE-containing chromatin (reviewed in [47–49]). Recent evidence from the maize genome highlights and furthers some of the advances made in understanding these regulatory processes. In the maize collection, Jia et al. evaluated the consequences of a deficiency in RNA-dependent RNA polymerase 2 (AtRDR2; mop1 in maize), a component of the RNA-directed DNA methylation silencing pathway [50]. Previous gene expression analyses suggested that even though *mop1* is expressed >100-fold higher in maize shoot apical meristems, the expression of some retrotransposons is substantially higher as well (when compared to seedlings) [51]. In this new analysis, the authors surveyed the expression changes in the *mop1* mutant for 797 DNA TE families and 608 retrotransposons to find that while most of the DNA TE families behaved as expected in a plant that is deficient in this silencing pathway (i.e., 78% of expression changes in DNA TE families was toward increased expression in the mutant), the retrotransposons behaved counter to expectations in that the expression changes observed were most often (68%) toward decreased expression [50]. In addition to changes in TE expression, many gene expression changes were also observed, most notably genes involved in chromatin modifications. Several histone deacetyltransferases, which have been implicated in heterochromatin formation in yeast [52], experienced increased expression in the *mop1* mutant indicating that those families experiencing decreased expression may be responding to increased heterochromatin formation in the mutant. The salient conclusion here is that not only are there multiple pathways and processes by which TEs are silenced, but these processes can interact and sometimes in an *antagonistic* fashion, which may permit relaxed control of some TE families and stricter control of other types. This highlights the complexities involved in TE regulation and impels a need for further exploration.

The recognition that TE silencing is RNAi based provides a reasonable explanation for the cessation of the transpositional bursts that shape genome size growth; as the copy number of a TE type increases, siRNAs derived from the new copies increase in number, which subsequently enhance silencing of that element type. Bursts of transposition are often considered to provide a temporary release from this or related forms of epigenetic suppression, a release suggested to be initiated by environmental stress [53–56] or an organismal process such as interspecific hybridization. In rice, for example, introgression has been linked to retrotransposon activation which was subsequently shutdown via cytosine methylation [57], while in sunflower, three independent hybridizations between the same two parents led to rapid proliferation of the same *gypsy* element in all three hybrid species [58, 59]. These and other studies lead to the widely held conceptualization that TEs are ever-present, typically "well-behaved" genomic residents being held in check through epigenetic suppression or by flying under the radar as low-copy elements, but which occasionally are "set loose" in different lineages and times in response to internal and external stresses that are not fully understood.

Recent work in *Arabidopsis* hints at a possible explanation for these periodic releases from suppression, while also underscoring the role that epigenetics plays with respect to TE deletion [60]. Based on the realization that methylated sequences often affect the expression of neighboring genes [61–63], the authors hypothesize that genes near to methylated TEs (met-TEs) would experience lower expression. In addition, if there exists a cost for methylating TEs that insert near genes, met-TEs would be subject to purifying selection and be more quickly removed from gene-rich regions. By analyzing the methylation status of TEs in the *Arabidopsis* genome, it was shown that TE methylation affects gene expression in a 1.5–2 kb window surrounding the gene. These results might help explain the differential deletion rates that exist for TE families, by suggesting that the insertion preferences and propensity for methylation characteristic of each family may influence the amount of negative selective pressure experienced by different types of elements. A second implication is that periodic releases from suppression may be a consequence of increased expression of a met-TE suppressed gene, achieved by TE demethylation under conditions of stress. Insight into the influence of epigenetic processes provided by this and similar studies on the various mechanisms that add to or eliminate DNA from genomes represent a key area for future research into genome size evolution.

4. Genetic Recombination and Genome Size

Because deletional mechanisms such as unequal intrastrand homologous recombination (UR) and illegitimate recombination (IR) are both recombination based, it stands to reason that local rates of genetic recombination might impact genome size evolution. Previous studies in rice [13],

Arabidopsis [12], and other species [15] have evaluated global rates of UR and IR; however, the effects of local rates of genetic recombination have been less frequently evaluated and results have been inconsistent. In *Arabidopsis lyrata*, a global bias exists between regions of differing recombination rates (i.e. TEs are more abundant in the gene-poor pericentromeric regions than in the more genic chromosome arms), but overall recombination rate does not correlate with TE abundance (when excluding pericentromeric-specific TEs) in intergenic space [64]. The authors suggest that the main factor influencing the differential association of TEs with pericentromeric and/or gene-poor regions is due to the short distances between genes in *A. lyrata* and the disruptive factor of TE insertions. A recent study in rice, however, suggests that the rate of genetic recombination can influence the rate of TE removal [65]. The authors evaluated the distribution and structural variation of LTR-retrotransposons (full-length, UR- or IR-deletion types) in the rice genome and related these to genomic features, including local rates of genetic recombination and gene density. Both the local rate of genetic recombination and gene density were negatively correlated with TE density, indicating that more TEs were allowed to accumulate in gene-poor regions of low recombination. In addition, UR recombination had the greatest effect in regions of high genetic recombination, whereas IR was most active in regions of low genetic recombination. Combined with their observation that UR is able to more quickly remove DNA than IR, the genomic balance and proportion of regions experiencing high or low rates of recombination may partly explain the differences each of these mechanisms have had historically in shaping genome size. That is, in species with relatively large areas of high recombination, UR will likely be the more active mechanism and will be responsible for rapidly removing DNA, whereas in species with large areas of low recombination, IR will be relatively more active and will be responsible for removing DNA at a slower rate. Furthermore, as recombination rates are fairly labile in plants [66], some of the differences observed in the rates of UR and IR between genomic regions and closely related species may be due to the average rate of genetic recombination for that region or species [16].

Surprisingly, the mechanisms of deletion are not the only ones associated with recombination. In an interesting discovery, Liu et al. uncovered a strong correlation between *Mutator* element genomic markers indicative of open chromatin, which is also associated with increased recombination. When examining *Mu* insertion site preferences, the authors found a nonrandom distribution that was similar to the patterns observed for recombination and gene density; that is, the rate of *Mu* insertions and recombination and gene density tend to be highest near the more euchromatic chromosome ends and then decrease as the distance to the centromere decreases [40]. Neither gene density nor a previously described preference for intragene insertion [67, 68] could adequately describe the pattern observed; however, upon comparison with existing cytosine methylation and histone modification data [69, 70], a strong association began to emerge. Both *Mu* insertions and recombination largely favored regions with strong signals for H3K4me3 and H3K9ac and with low levels of cytosine methylation, all suggestive of open chromatin structure. The authors suggest that other TEs that display biases toward genic regions (e.g., Ac/Ds or MITEs) may also rely on open chromatin structure for insertion. Thus, while the distribution of *Mu* insertions does not rely on recombination rate, per se, recombination rate itself may be indicative of regions susceptible to open chromatin targeting TEs. As the aforementioned lability in recombination rates may mirror a similar lability in chromatin structure, the success of these types of elements may be influenced by differences among species. Clearly more data are needed to gain a clearer perspective on the effect of recombination and chromatin structure on TE success and persistence.

5. Population Genetics and Genome Size

Discussions concerning genome size evolution often center on the mechanisms, events or rates of change that historically have influenced the genome size of a given lineage or a set of taxa, often using one individual per species. Population-level processes such as effective population size and breeding system may also contribute to the shaping of genomes, though at present few studies address this relationship. Because most TE insertions that survive are neutral to slightly deleterious, they are subject to the twin processes of selection and drift, and thus relative levels of TE survival are contingent not just on their internal, genomic ecology but also on external, population level forces. Much of the empirical evidence (from mostly bacterial and animal systems) and relevant theory is presented in Lynch's recent book *The Origins of Genome Architecture* [71]. This relatively new area of research is now attracting interest from plant biologists.

Illustrative of this approach, Hollister and Gaut detail the influence of population dynamics on the retention of a Helitron element, *Basho*, in the genome of the selfing plant, *Arabidopsis thaliana.* Using a subset (278 of 565) of the *Basho* elements predicted in the *A. thaliana* genome, they screened a diverse panel of 47 accessions to determine the frequency of Helitron occupation at each insertion site and related these to genomic factors such as element length, proximity to genes, and recombination rate. A high rate of fixation was detected, as compared to an analogous study in *Drosophila* [72, 73], with nearly 50% of the evaluated elements achieving fixation and 81% existing in over half of the accessions surveyed, consistent with the notion that genetic drift in this inbred plant permitted the fixation of presumably slightly deleterious sequences. The authors also found that the age of an element is strongly and positively correlated with fixation, as expected for neutral alleles, whereas length and proximity to genes is negatively correlated with fixation. Thus, selection against element accumulation is likely weak and dependent upon the size of the element, due to the potentially deleterious effects of a greater potential for ectopic recombination in longer sequences. The authors suggest further that ectopic recombination is important in governing the persistence of *Basho* in the *A. thaliana* genome

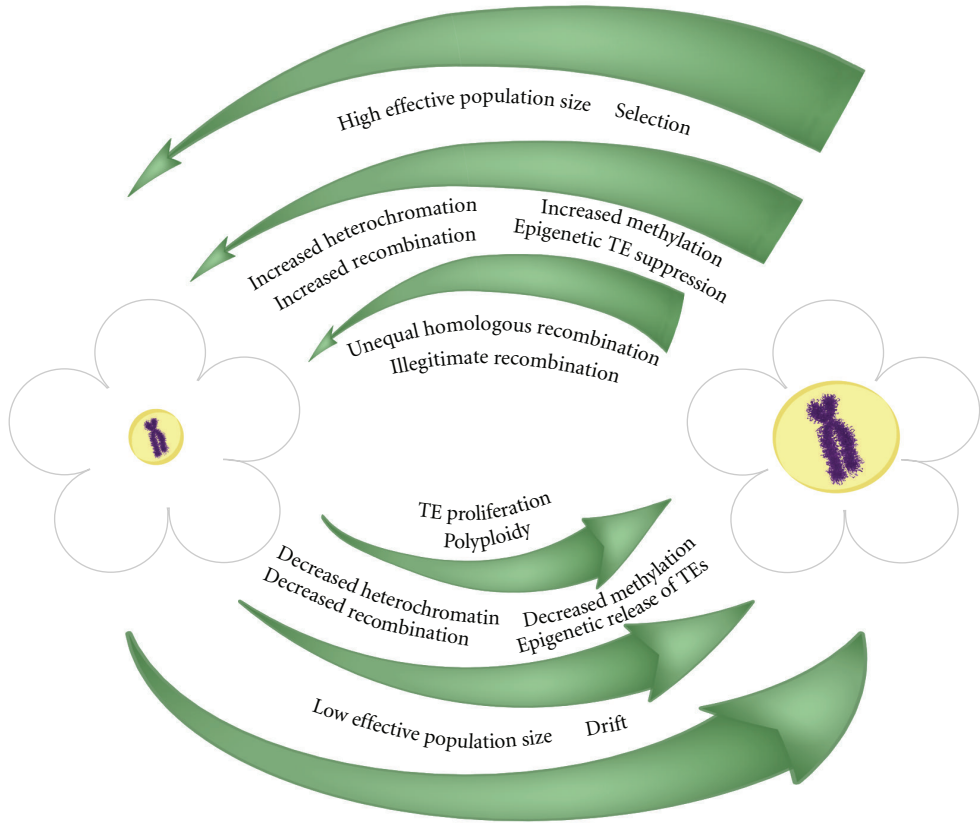

Figure 1: The effects that various mechanisms, genomic properties, and population influences exert on genome size. The innermost ring represents proximal mechanisms responsible for generating genome size differences, while the second and third rings represent genomic properties and population size effects, respectively, which affect the magnitude of those mechanisms.

and suggest that the weak selection observed in *A. thaliana* may be higher in an outcrossing relative (e.g., *A. lyrata*) where the heterozygous state of many TEs would provide more potential for ectopic pairings.

In a similar investigation, Lockton et al. used transposon display for six diverse families of TEs (*Gypsy*-like LTR-retrotransposon; SINE-like and LINE-like non-LTR-retroelements; Ac-like, CACTA-like, and *Tourist*-like DNA elements) to generate polymorphism datasets for five populations of *Arabidopsis lyrata* that had previously been described demographically [74, 75]. Interestingly, individual TE bands were found in intermediate to high population frequencies, suggesting that the selection has not strongly been operated to remove these TEs. Because mean TE "allele" frequencies are lower in *A. lyrata* than in *A. thaliana* (24% versus 60% across TE families), the data are consistent with the expectation that TEs in an outcrossing species experience stronger selection and are less subject to drift. An interesting added dimension of this analysis was the calculation of selection coefficients for the individual populations (located in Sweden, Iceland, Russia, the United States, and Canada, and a larger refugial population located in Germany). Most of the populations, save the refugial German population, had positive estimates of selection, counter to the expectation of purifying (negative) selection against TEs. They inferred that in these populations, all of which had significantly lower effective population sizes than the larger German population (by 7–18 fold), drift is able to overcome the weak selection against TE insertions experienced in A. *lyrata* (as estimated using the larger German population). One important implication is their suggestion, based on these data, that the rate of genomic flux in TEs is influenced not only by current effective population size and breeding system, but also by demographic history. Thus, small population sizes, inbreeding, and population bottlenecks are all conditions that lead to a less effective environment for purging TEs, and hence genome sizes might be predicted to expand more rapidly than comparable plant populations not experiencing these conditions (all else being equal).

6. The Future of Genome Size

Our understanding of the mechanisms responsible for genome size evolution has vastly improved over the past decade, with a number of reviews devoted to the patterns exhibited by these mechanisms among a variety of species [5, 6, 76, 77]. Less discussed and only more recently addressed are the multiple factors that influence insertional and deletional processes as well as their context-dependent interactions. Thus, for example, we have only begun to explore how genomic properties, such as recombination and

epigenetic context, and population level processes, including effective size, history, and breeding system, affect short-term and longer-term genome size evolution (Figure 1). As next-generation sequencing technologies become more accessible and increasingly applied, it is now possible to design studies that will enhance our understanding of these many interactions. Incorporating analyses across several phylogenetic scales will yield insights into the forces that shape modern plant genomes and help explain their current diversity and distinctions.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 341380, 12 pages
doi:10.1155/2010/341380

Review Article

Genome Size in Diploids, Allopolyploids, and Autopolyploids of Mediterranean Triticeae

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Received 12 January 2010; Accepted 29 April 2010

Academic Editor: Jaroslav Doležel

Nuclear DNA amount, determined by the flow cytometry method, in diploids, natural and synthetic allopolyploids, and natural and synthetic autopolyploids of the tribe Triticeae (Poaceae) is reviewed here and discussed. In contrast to the very small and nonsignificant variation in nuclear DNA amount that was found at the intraspecific level, the variation at the interspecific level is very large. Evidently changes in genome size are either the cause or the result of speciation. Typical autopolyploids had the expected additive DNA amount of their diploid parents, whereas natural and synthetic cytologically diploidized autopolyploids and natural and synthetic allopolyploids had significantly less DNA than the sum of their parents. Thus, genome downsizing, occurring during or immediately after the formation of these polyploids, provides the physical basis for their cytological diploidization, that is, diploid-like meiotic behavior. Possible mechanisms that are involved in genome downsizing and the biological significance of this phenomenon are discussed.

1. Introduction

The study of nuclear DNA amount (C-value in pg) in many plant groups during the last several decades not only yielded data on nuclear DNA amount in a large number of plants (see the Plant DNA C-values Database at Royal Botanic Gardens, Kew, available from http://data.kew.org/cvalues/), but has also contributed to a variety of related studies such as ploidy screening, detection of aneuploids, cell cycle kinetics, and reproductive pathways [1] and, through this, to several scientific disciplines including systematics, cytogenetics, and evolution [2]. The understanding that related species may differ in genome size and that these differences result primarily from changes in the amount of noncoding DNA [3] has motivated studies on the genetic and evolutionary significance of these differences, on the patterns of genome size changes in different plant groups, and on mechanisms underlying genome expansion or contraction.

The determination of nuclear DNA amount in many plant species has been performed with different methods, some of which are outdated and less accurate than flow cytometry [1–5]. Consequently, data obtained by different scientists using different methods for the same species were inconsistent. Therefore, as yet, there is no clear-cut knowledge as to the degree of variation in genome size neither at the intra-specific level nor for the occurrence and extent of genome downsizing in allopolyploids and autopolyploids.

One of the intriguing issues concerning evolutionary dynamics of plant genomes is the extent of intra-specific variation in genome size. Several previous studies have shown that there is a significant variation in genome size at the intra-specific level of several plant species, for example, *Dasypyrum villosum* [6], *Arachis hypogaea* [7], and *Hordeum spontaneum* [8]. However, these findings were questioned in several cases by reinvestigation with an improved technical approach [9].

Using the DNA C-values database, Leitch and Bennett [10] found that many polyploids from diverged groups of species showed a reduction in nuclear DNA amount relative to that of the respective diploids and that the mean DNA amount per basic genome (DNA amount divided by the level of ploidy) tends to decrease with increasing ploidy. They concluded that genome downsizing following polyploid

formation is a widespread phenomenon. However, Leitch and Bennett [10] also noted that in most cases the precise parental genome donors of the studied polyploid species were unknown and, therefore, many comparisons between polyploids and their putative diploids were inappropriate. Despite the study of Leitch and Bennett [10], there is still conflicting evidence in different plant groups concerning the question whether the C-value of a polyploid is less than the sum of the C-values of its diploid parents (e.g., [11–14]) and, therefore, this question deserves further consideration. Also, Leitch and Bennett [10] did not distinguish between allopolyploids and autopolyploids; the latter might contain the expected additive amount of the nuclear DNA of their diploid parents [15–17]. The data of the Plant DNA C-values Database at Royal Botanic Gardens, Kew (available from http://data.kew.org/cvalues/), show that while several autopolyploids contain the expected additive DNA amount others contain smaller amount of DNA than expected. Hence, the effect of autopolyploidization on genome size is not clear and has not been studied adequately.

Recently, using flow cytometry, Eilam et al. [18–20] studied these topics in species of the tribe Triticeae (Poaceae). The species of this tribe exhibit large differences in their genome size with nuclear DNA amount ranging from 4.0 pg per 1C nucleus in *Elymus libanotica* to 8.3 pg in *Secale cereale* [21, 22]. Since these differences may reflect genome complexity that bears genetic and evolutionary meaning, it was of interest to determine the ranges of intra-specific variation in genome size in these species and to see to what extent allopolyploidy and autopolyploidy affect genome size. This paper reviews recent data on genome size in diploid, allopolyploid, typical autopolyploid, and cytologically diploidized autopolyploid taxa of Mediterranean Triticeae [18–20].

The tribe Triticeae of the family Poaceae includes 16 genera comprising several hundreds of species that grow mainly in the temperate-arctic and Mediterranean—central Asiatic regions of the northern hemisphere. Several evolutionary trends have been recognized in this tribe: the primitive genera contain perennial species that have spikelets in groups at each rachis internode and grow mainly in the temperate-arctic region while the more advanced genera contain annual species that have solitary spikelets at each rachis internode and grow mainly in the Mediteranneancentral Asiatic regions [23]. Another significant evolutionary trend in this tribe is the prevalence of polyploidy (allopolyploidy and autopolyploidy) in most of the genera: about 31% of the species are diploids and 69% are polyploids in this tribe. The evolutionary success of allopolyploids and autopolyploids stems from a different genetic system. Allopolyploids, derived from inter-specific and intergeneric hybridization followed by chromosome doubling, have two or more diverged (homoeologous) genomes. These genomes usually undergo further divergence resulting in cytological diploidization and consequently, diploid-like meiotic behavior that leads to full fertility and disomic inheritance [24–26]. Such a genetic system, reinforced by predominant self-pollination, may result in true breeding and permanent maintenance of favorable intergenomic genetic interactions between homoeoalleles [26]. Autopolyploids, on the other hand, originated mostly from intra-specific hybridization followed by chromosome doubling, have multiplicity of homologous genomes. Thus, their chromosomes may form multivalents during meiosis leading to a multisomic inheritance. One advantage of autopolyploidy is the capability to maintain different degrees of intralocus heterozygosity, or more rarely, to reach homozygosity with multiple dosage of a given allele. Thus, in spite of the fact that multivalent pairing is often associated with some degree of sterility, selection in some autopolyploids will favor multivalent formation, and therefore will act against genomic changes that may lead to cytological and genetic diploidization. Nevertheless, in addition to these "typical autopolyploids", there are many autopolyploids that underwent cytological and genetic diploidization and consequently form only or mostly bivalents at meiosis. These "cytologically diploidized autopolyploids" [20] may have multisomic inheritance if the bivalents are formed at random between the four homologues, or may have disomic inheritance if the bivalents are formed always between the same partners as a result of preferential pairing.

The taxonomy of the Triticeae in this paper follows that of Clayton and Renvoize [27] and that of the *Aegilops-Triticum* species follows that of Eig [28] and van Slageren [29]. Genome designation of the Triticeae species is after Wang et al. [30] and that of *Aegilops* and *Triticum is* after Kimber and Tsunewaki [31].

2. Genome Size at the Intraspecific and Interspecific Levels

The nuclear 1C DNA amount in diploid and allopolyploid Triticeae species and in diploid and autopolyploid Triticeae cytotypes exhibits very small variation between plants of the same line just as the variation between lines (Table 1; see also [18, Tables 1 and 2], [19, Tables 1 and 2] and [20, Tables 1 and 3]). The analysis of variance revealed that most variation (>92%) was due to species or cytotypes whereas between plants and between lines variation contributed together to the total variation only a small fraction of this value. This intra-specific or intracytotype stability in genome size was found among lines that were collected from different parts of the species' geographical distributions as well as from different habitats. Different morphs of each species such as small and large types of wild barley that were collected from different localities did not differ in their nuclear DNA amount. Also there were no significant differences in nuclear DNA amount of lines that were collected from different habitats (e.g., dry versus humid, hot versus cold, and peripheral versus central). No significant difference in DNA amount was found between lines of wild and domesticated diploid and tetraploid wheat and between wild (*H. spontaneum*) and domesticated (*H. vulgare*) barley. The intra-specific and intracytotype stability in nuclear DNA amount indicates that there were very little changes, if any, in DNA amount during the life history of the diploids, allopolyploids, and autopolyploids.

Table 1: Analysis of variance and variance components estimates of DNA content in different lines of 22 diploid Triticeae species (a), in different lines of 24 allopolyploid species of *Aegilops* and *Triticum* (b), and in different lines of diploid and autotetraploid cytotypes of *Elymus* and *Hordeum* (c) analyzed in a random effect model.

(a) Diploid species.

Source of variation	df	Sum of squares	Mean square	F	Variance components	Percent of total variance
Species	22	288.056	13.0935	1049.204*	0.878816	98.814
Lines (Species)	249	3.123	0.01254	1.8292*	0.003693	0.415
Plants (Lines, Species)	166	1.138	0.00686	—	0.006857	0.771
Total					0.889366	100.000

(b) Allotetraploid and allohexaploid species.

Group	Source of variation	df	Sum of squares	Mean square	F	Variance components	Percent of total variance
Tetraploids	Species	18	150.087	8.3382	80.444**	1.05737	90.97
	Lines (Species)	64	6.650	0.1039	0.979 n.s.	−0.00107	−0.09
	Plants (Lines, Species)	95	10.080	0.1061	—	0.10610	9.13
	Total					1.16246	100.00
Hexaploids	Species	6	4.232	0.7053	5.168*	0.14267	61.73
	Lines (Species)	14	1.720	0.1228	3.664*	0.05492	23.76
	Plants (Lines, Species)	16	0.536	0.0335	—	0.03352	14.51
	Total					0.23111	100.00

(c) Diploid and autotetraploid cytotypes.

Group	Source of variation	df	Sum of squares	Mean square	F	Variance components	Percent of total variance
Diploids	Cytotypes	3	5.725	1.9082	94.649*	0.54589	97.03
	Lines (Cytotypes)	11	0.261	0.0238	7.895*	0.01373	2.44
	Plants (Lines, Cytotypes)	24	0.078	0.0032	1.641	0.00113	0.20
	Residual					0.01850	0.33
	Total					0.56261	100.00
Tetraploids	Cytotypes	4	35.870	8.9676	101.618*	0.06829	56.63
	Lines (Cytotypes)	30	2.893	0.0964	8.671*	0.03500	27.49
	Plants (Lines, Cytotypes)	37	0.394	0.0106	0.576	0.00009	0.07
	Residual					0.02396	18.82
	Total					0.12735	100.00

*$P \leq .01$; **$P \leq .001$.

The great stability observed by Eilam et al. [18–20] in nuclear DNA amount at the intra-specific level of every studied Triticeae species is in accord with a large number of reports indicating lack of significant variation in genome size at the intra-specific level of other plant groups [1, 9, 16, 32–41]. These studies could not confirm previous observations (e.g., [6–8]) of intra-specific variation in genome size that were reported in several species. Hence, the data of Eilam et al. [18–20] support the notion that intra-specific variation in genome size is relatively small and non-significant [9, 42, 43].

In contrast to the stability of genome size at the intra-specific level, there is a large and significant variation in this trait at the inter-specific and the intergeneric levels

of the studied Triticeae species (Table 1; see also [18–20]). No evidence was found in these studies indicating that self-pollinating species (most of the species) have considerably different nuclear DNA amounts than the cross-pollinating species. Similarly, the values of DNA amounts of the perennial species fell within the range of values of the annual species (only *Secale montanum* has larger genome than its annual relatives). This is in accord with the data of Albach and Greilhuber [44] in *Veronica* species and Jakob et al. [16] in *Hordeum* species and in contrast to previous reports [45, 46] claiming that annual, self-pollinating species have smaller genomes than perennial, cross-pollinating ones. Diploid species that grow in the southern part of the distribution area of the genus *Aegilops*, that is, in hotter and drier habitats (*Aegilops bicornis*, *Ae. searsii, Ae. Longissima,* and *Ae. sharonensis*), have significantly more DNA than species that grow in other parts of the distribution of *Aegilops*.

The genome-size stability at the intra-specific level of the Triticeae species studied here is striking in view of the fact that retrotransposons comprise a significant fraction of the genomes of many Triticeae species [47–49] and, as such, these genomes have a considerable potential to undergo rapid changes in nuclear DNA amount.

As found for the grass family in general [50], in the Triticeae too, there is no clear trend of genome-size evolution on the inter-specific level; in some species genome was increased while in others it was decreased. Increase in genome size can be brought about by the activation of transposons, especially retrotransposons [51–54], whereas decrease in DNA amount can result from a variety of recombinational mechanisms such as unequal homologous recombination between homologous chromosomes, sister chromatids, or intrachromatid; the latter can take place between long terminal repeats (LTRs) of retrotransposons [53, 55–57]. Decrease in DNA may also result from illegitimate recombination between chromosomes, sister chromatids, or intrachromatid [53, 57]. The relative extent of these two counteracting mechanisms (retroelement proliferation and LTR recombination) determines the direction of genome-size change. The LTRs of a single retrotransposon diverge from one another with time, thus limiting the possibility of intrachromatid recombination. Consequently, old retrotransposons can only increase DNA amount by activation and transposition rather than decrease it by illegitimate intrachromatid recombination.

The inter-specific differences in genome size reflect mostly variation in the noncoding DNA since grass genomes contain a similar number of genes [58] and differences in genome size are mainly related to differences in transposable elements content [3, 59, 60]. The contrast between the low variation at the intra-specific level and the high variation at the inter-specific one (Table 1) may suggest that the inter-specific changes occurred during speciation. In other words, speciation within many Triticeae genera was either accompanied by or even resulted from a considerable change in nuclear DNA amount. Such a change could have been induced by environmental stress such as sudden heat, frost, and drought [61, 62], or genetic stress such as inter-specific hybridization [63].

3. Genome Downsizing in Allopolyploids

The wheat group (the genera *Aegilops* and *Triticum*) is most suitable for studying genome size in allopolyploids. It contains many allotetraploid and allohexaploid species that, because of their economic importance, have been subjected to an intensive cytogenetic study that has provided considerable information on the origin of their genomes and on the genomic relationships among them [64]. The amount of nuclear DNA was recently estimated in the diploid species of this group [18] and determination of nuclear DNA amount in the allopolyploids facilitated comparisons between the allopolyploids and their progenitors [19, 20]. Moreover, it is rather easy to produce synthetic allopolyploids in this group and many such newly formed allopolyploids were recently synthesized [65]. In such material, comparisons were made not only between synthetic allopolyploids and their parental plants but also between synthetic and natural allopolyploids having the same genomic combination.

The nuclear 1C DNA amount in the allopolyploids of the wheat group was determined using flow cytometry [19]. The expected amount of nuclear DNA in each of the allotetraploid species was calculated from the sum of the DNA amount in their two parental diploid species and that in allohexaploid species from the sum of the DNA amount in the respective tetraploid and diploid parental species. The amount of DNA in most allopolyploid species was significantly smaller than the expected value ([19, Table 1] and Figure 1). The data of Eilam et al. [19] contradict several previous studies [12, 66–70] which measured DNA amount by staining the nuclei with the DNA-specific Feulgen stain followed by quantifying the absorption of the visible monochromatic light. They showed that the nuclear DNA amount in *Aegilops* and *Triticum* allopolyploids is the sum of the DNA amount of their parental species. The discrepancy between these results and those of Eilam et al. [19] may be explained by the method of estimation of DNA amount. Eilam et al. [19] estimated nuclear DNA amount by flow cytometry that is a more accurate and precise method [1], in a relatively large number of plants in each species and in a large number of nuclei in every plant, each plant in several replicates. The accuracy of the method used by Eilam et al. [19] is evident from the non-significant differences that were obtained between replicates. Moreover, the results of Eilam et al. [19] are in accord with those of Pai et al. [71] and Upadhya and Swaminathan [72] who found that DNA content in hexaploid wheat is less than expected from the amount of its parental species. Similarly, Boyko et al. [73, 74] found a decrease in DNA amount of octoploid and hexaploid triticale (*Triticosecale*), respectively, as compared to the combined wheat and rye (*Secale cereale*) values. All these data are in accord with recent findings that allopolyploidization leads to instantaneous elimination of DNA sequences in the wheat group [24, 65, 75–81] and in triticale [82–84].

The results of Eilam et al. [19] show that the allotetraploid species of *Triticum*, *T. turgidum* (genome BBAA), had larger amount of DNA than the expected value which was calculated on the assumption that *Ae. speltoides* is the donor of the B genome and *T. urartu* is the donor of

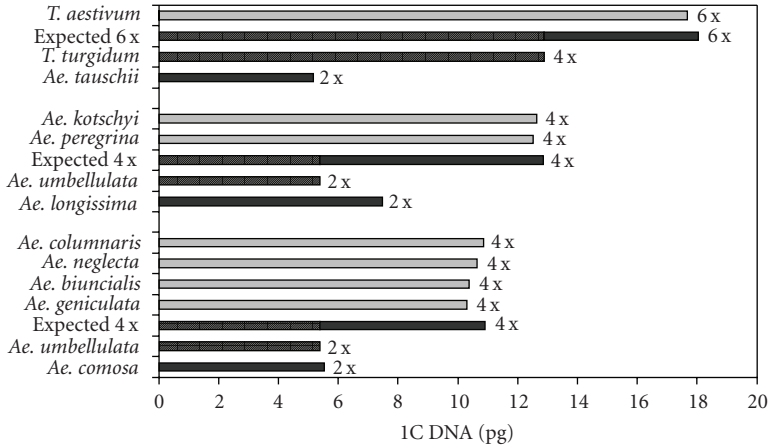

Figure 1: Expected (black-stripped bars) and observed (gray-solid bars) nuclear 1C DNA amount in natural *Aegilops* allotetraploids ($2n = 4x = 28$) having the UM or US genomes and *Triticum* allohexaploid ($2n = 6x = 42$) having the BAD genome (data from [19]). Nuclear 1C DNA amount of the putative parental species is also presented. The differences between the expected and observed were significant (*t*-test; $P \leq .05$) except those in *Ae. neglecta* and *Ae. columnaris* (in these two species a very small number of lines were analyzed).

the A genome. The deviation from the expected amount is very large and significant. Similar results were obtained by Furuta et al. [85] who found that the DNA content of *Ae. speltoides* is only about 87% of that of the B genome of hexaploid wheat and therefore concluded that *Ae. speltoides* is not the donor of the B genome. Hence, the B genome was either derived from a species containing more than 7.5 pg DNA or, if it is derived from *Ae. speltoides,* its DNA amount was increased in the tetraploid wheat. If so, the B genome is unique in undergoing increase, as all other genomes of *Aegilops* and *Triticum* allopolyploids underwent downsizing, including the synthetic allopolyploids containing the S genome of *Ae. speltoides* (Figures 1 and 2 and [19]). The decrease in synthetic allpolyploids containing the S genome is in accord with recent reports [79, 81] describing elimination of repetitive sequences from genome S of *Ae. speltoides* in newly formed allopolyploids.

The nuclear DNA amount of the newly formed allopolyploids and of their parental plants is presented in Figure 2. Eight out of ten synthetic allopolyploids contain significantly less DNA than the expected additive sum of DNA amounts of their two parental plants. The decrease in DNA amount is already evident in the first generations following chromosome doubling and remains fairly similar in subsequent generations. Comparison of nuclear DNA amount in synthetic and natural allopolyploids having the same genomic combination showed non-significant differences between them (Figure 3). These results are in accord with those of Ozkan et al. [86] who found in six newly synthesized wheat allohexaploids that they have a genome size significantly smaller than the expected additive value of their parental plants. Decrease in genome size occurred already in the first generation of the allopolyploids, indicating that the change was a rapid event [86]. This corresponds to the data of Ozkan et al. [65], Shaked et al. [78], and Ma and Gustafson [82] that elimination of DNA sequences occurs in the $\mathbf{F_1}$ hybrid and in the first generation(s) of the newly formed allopolyploids. The low variation in nuclear DNA amount at the intra-specific level (Table 1) suggests that the changes in genome size occurred soon after the formation of the allopolyploids and no further significant changes occur during the life history of the allopolyploids. The similarity in DNA values of natural and synthetic allopolyploids having the same genomic combinations (Figure 3) also shows that changes in DNA amount were reproducible and occurred immediately after the formation of the allopolyploids. An interesting question is, therefore, what stabilizes genome size in these allopolyploid species that contain large amount of transposable elements [47] that may, as a response to external or internal stimulation, increase or decrease nuclear DNA amount.

There is evidence indicating that the decrease in DNA amount in allopolyploids was not equal in the different constituent genomes and one genome underwent more drastic downsizing than the other(s). Extracted tetraploid wheat, having the B and A genomes of the allohexaploid wheat *T. aestivum* [87, 88], contains similar amount of nuclear DNA as the natural BBAA allotetraploid *T. turgidum* (Figure 4). Also Furuta et al. [70], Nishikawa and Furuta [89], and Nishikawa et al. [90] found that DNA content of BBAA genome component extracted from three cultivars of *T. aestivum* is equal to that of *T. turgidum*, indicating that DNA amount of the B and A genomes of *T. aestivum* did not change at the hexaploid level during 10,000 years of history of this wheat. It can be deduced, therefore, that the reduction in DNA amount in natural and synthetic allohexaploid wheat found by Eilam et al. [19] was mainly in the D genome. Consequently, the amount of nuclear DNA in the D genome of *T. aestivum* is 4.96 pg (17.67 pg in *T. aestivum* subsp. *aestivum* minus 12.91 pg in the extracted tetraploid), that is, 11% smaller than that of *Ae. tauschii,* the diploid donor of the D genome to *T. aestivum*. Similarly,

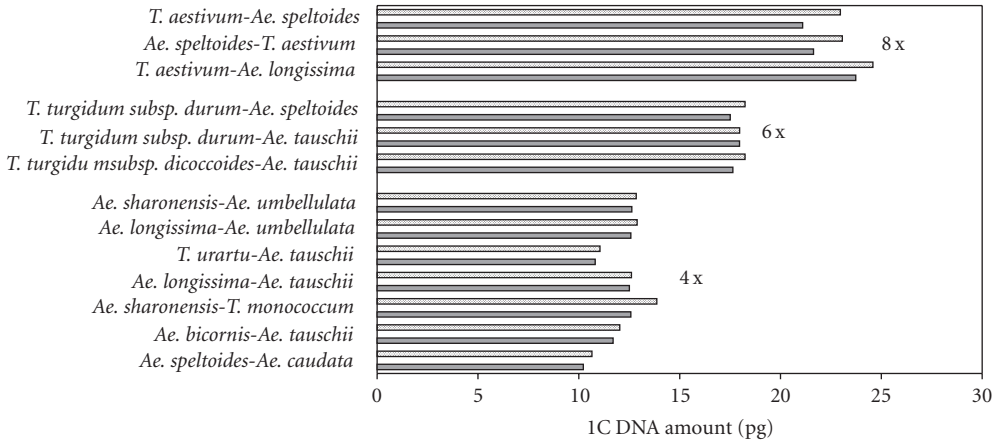

Figure 2: Nuclear 1C DNA amount in 4x, 6x, and 8x synthetic allopolyploids of *Aegilops and Triticum* (gray-solid bars) and DNA values expected from their parental plants' value (black-dotted bars) (data from [19]). The differences between expected and observed were significant (t test; $P \leq .05$) in all synthetic allopolyploids.

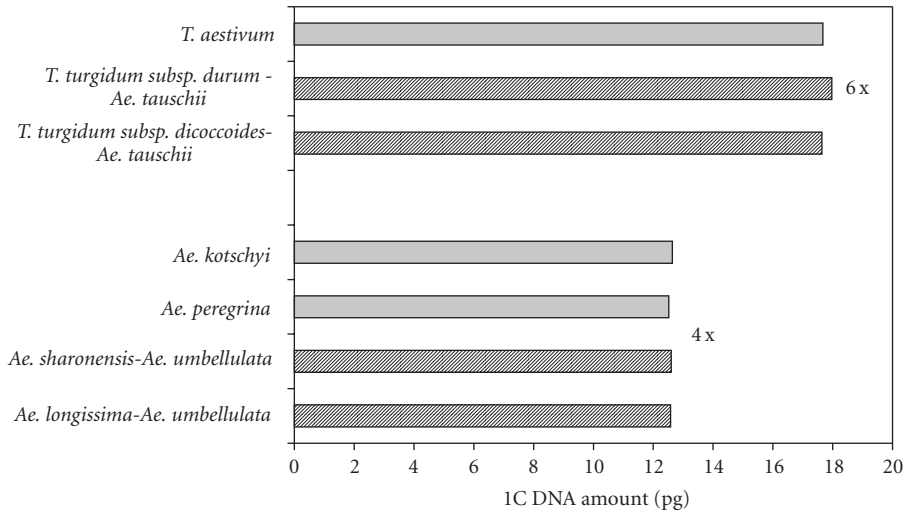

Figure 3: Nuclear 1C DNA amount in natural (gray-solid bars) and synthetic (black-reticulated bars) allopolyploids having similar genomic combinations (data from reference [19]). The differences between natural and synthetic are not significant (t-test; $P \leq .05$).

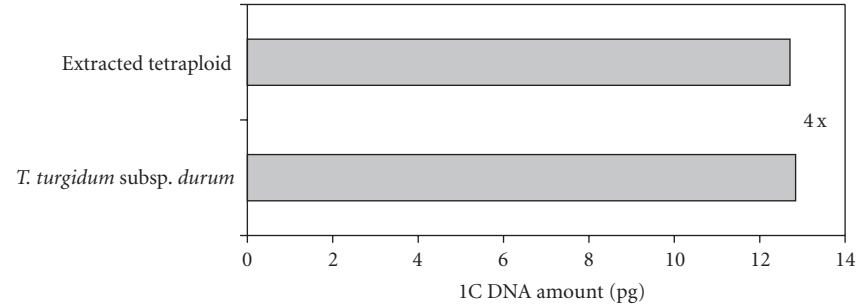

Figure 4: Nuclear 1C DNA amount in extracted tetraploid wheat ($2n = 4x = 28$) having the B and A genomes of hexaploid wheat *T. aestivum*, and in tetraploid *T. turgidum* subsp. *durum* ($2n = 4x = 28$; genome BBAA) (data from [19]). No significant difference exists between the 1C DNA amounts of the two taxa.

Furuta et al. [70] found that the D genome of the newly synthesized *T. aestivum* contains 4.39 pg DNA, 14.4% less than in *Ae. tauschii*. It is assumed that the B and A genomes underwent genome downsizing already at the tetraploid level and, therefore, did not change further at the hexaploid level. Likewise, Ma and Gustafson [83] described a differential decrease in DNA amount in the constituent genomes of a newly synthesized allopolyploid. They found in octoploid triticale a greater loss of DNA sequences from the rye genome than from the wheat ones.

The results described above are in accord with those of Leitch and Bennett [10] who found that genome downsizing characterizes wide groups of allopolyploids. Recent studies, for example, Vaio et al. [91] in allotetraploid *Paspalum* species, Johnston et al. [92] in Brassicaceae allopolyploid species, Vogel et al. [15] in Triticeae allopolyploid perennial species, and Pires et al. [13] in allotetraploid, *Tragopogon miscellus*, also show that genome downsizing in allopolyploids is a very prevalent phenomenon. Thus, genome downsizing, being a general response to allopolyploid formation, may represent an overall trend in allopolyploid evolution across the angiosperms [10].

4. Genome Size in Autopolyploids

Two types of autopolyploids are recognized in the Triticeae: "typical" and "cytologically diploidized" autopolyploids. Typical autopolyploids, represented by the tetraploid cytotype of *Hordeum bulbosum* L., are characterized by multivalent pairing at meiosis, four to five multivalents per meiocyte [93–95], and, consequently, multisomic inheritance. The formation of multivalents during meiosis is often associated with partial sterility. Thus, typical autopolyploids are prevalent among perennial species having the capacity for vegetative propagation in addition to the sexual one. Cytologically diploidized autopolyploids, represented by the tetraploid cytotype of *Elymus elongatus* (Host) Runemark [=*Agropyron elongatum* (Host) P. Beauv.], exhibit exclusive or almost exclusive bivalent pairing at meiosis in spite of the fact that originally they contained two or more homologous genomes [96–98]. The cytologically diploidized autopolyploids, being more fertile than the typical ones, are prevalent among annual species. The genus *Hordeum* contains several species that include tetraploid cytotypes exhibiting exclusive bivalent pairing at meiosis [99]. These cytotypes are either cytologically diploidized autopolyploids or segmental allopolyploids [16, 100, 101]. Many of these species are annuals lacking vegetative reproduction.

The nuclear DNA amount in diploid and tetraploid cytotypes of *Hordeum bulbosum* was determined [20]. It was found that this typical autopolyploid contains the expected additive DNA amount (see [20, Table 1]). Similarly, Vogel et al. [15] and Jakob et al. [16] found that the nuclear DNA amount of this autotetraploid cytotype does not deviate significantly from the expected additive value of its parental diploid cytotype. Likewise, the typical autotetraploid cytotype of *Dactylis glomerata* and autopolyploids of the genus *Festuca* (all exhibit multivalent pairing) contained the expected additive DNA content of their parental species [102]. Also tetraploid cytotypes of *Allium* species [103], newly induced autotetraploid *Brassica* plants [104], and an induced autotetraploid line of *Festuca pratensis* [105] showed no significant difference between the expected additive amount and observed DNA content. Similarly, Ozkan et al. [17], studying genome size in two newly formed autotetraploid accessions of *Arabidopsis thaliana*, found that they contained the expected additive amount of nuclear DNA. From the above it can be concluded that typical autopolyploids have the expected additive nuclear DNA amount of their parental diploids. The fact that no genome downsizing has occurred in these taxa indicates that the homology between the homologous chromosomes of the constituent genomes is maintained, thus facilitating pairing between all homologous chromosomes and consequently formation of multivalents at meiosis.

In contrast to the additive value of genome size in typical autopolyploids, nuclear DNA amount in cytologically diploidized autotetraploids exhibits considerable genome downsizing. The nuclear DNA amount was determined in diploid and autotetraploid cytotypes of *E. elongatus* and in diploid and autotetraploid cytotypes of several *Hordeum* species [20]. Evidently, the tetraploid cytotypes contained much less DNA than the expected additive amount of the diploid cytotypes (Figures 5 and 6). The nuclear DNA amount in a newly synthesized autotetraploid line of *E. elongatus* was found to be significantly smaller than the expected additive value of the diploid parental plant (Figure 5). Comparison of the nuclear DNA amount in the synthetic and the natural autotetraploid cytotypes of *E. elongatus* showed small and non-significant difference between them (Figure 5), thus indicating that genome downsizing in this autopolyploid was reproducible and occurred immediately after polyploidization and there were no further changes in genome size during its life history.

The cytologically diploidized autotetraploid *H. murinum* subsp. *murinum* and the segmental allotetraploid *H. murinum* subsp. *leporinum* also had significantly less nuclear DNA than the expected value (Figure 6). Similar results were obtained by Raina et al. [106] who described decrease in DNA amount in newly synthesized autotetraploid *Phlox*: DNA amount was decreased by 16.7% in C_0 and by 25% in C_2 generation. The loss was the outcome of equal reduction in all of the chromosomes. Rapid DNA loss in the first and second generations was associated with increased seed set and also helped in the establishment and stabilization of the autotetraploid [106].

The cytologically diploidized autotetraploids studied here form exclusively bivalents at meiosis [97–99, 107, 108]. The shift from potentially multivalent pairing towards bivalent type of pairing in these autopolyploids presumably was brought about by instantaneous elimination of DNA sequences that are involved in homology recognition and initiation of meiotic pairing from two out of the four homologous chromosomes in each set of four, several sequences from one pair and other sequences from the second pair. Indeed, Dvorak [109] noticed that the two genomes of the tetraploid cytotype of *E. elongatus* appeared

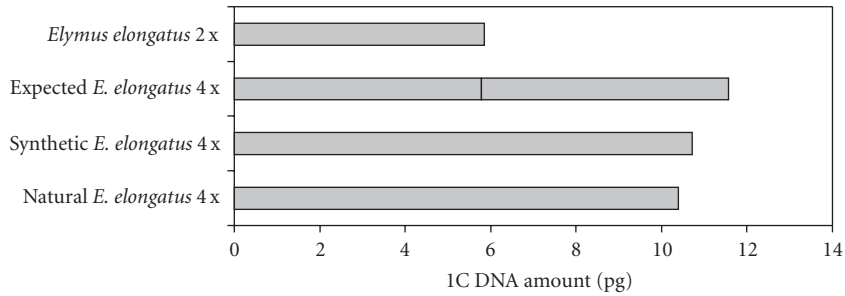

Figure 5: Expected and observed nuclear 1C DNA amount in natural and synthetic autotetraploid *Elymus elongatus* and in the diploid parental plant of the synthetic autotetraploid (data from [20]).

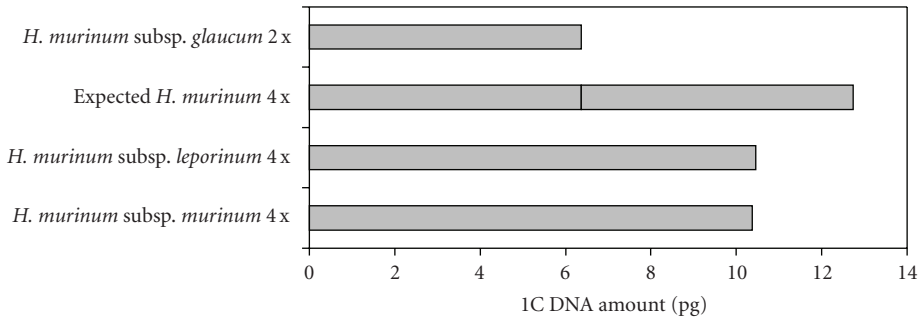

Figure 6: Expected and observed nuclear 1C DNA amount in diploid and natural autotetraploid subspecies of *Hordeum murinum* (data from [20]).

to be a modified version of the diploid genome. Since the two genomes are slightly diverged, pairing between fully homologous chromosomes, that is, intra-genomic pairing, leads to bivalents at meiosis and disomic inheritance [109].

The data presented here indicate that the bivalent pattern of pairing exhibited by the tetraploid cytotypes of *E. elongatus*, *H.murinum* subsp. *Murinum,* and *H. murinum* subsp. *leporinum* may have resulted from instantaneous elimination of DNA sequences—several sequences from one of the genomes and others from the second genome—thus augmenting the divergence between the homologous chromosomes of the different genomes. A gene system, determining diploid-like meiotic behavior, may be superimposed on sequence elimination, taking advantage of the divergence between the homoeologues of the different genomes, and, consequently, allowing strict pairing between the same partners with the resultant disomic inheritance. Fixed heterozygosity caused by disomic inheritance was seen in the segmental allopolyploid *H. murinum* subsp. *leporinum* [110–113] and in the cytologically diploidized autotetraploid *H. murinum* subsp. *murinum* [114, 115]. Thus, this group of autopolyploids benefit from full fertility and permanent fixing of heterozygosity between alleles of the partly diverged genomes.

In summation, genome downsizing in autopolyploids might bring about their cytological diploidization, facilitating their rapid and successful establishment in nature, increasing their competition with the parental species, and improving their adaptability to new environments. The decrease in DNA content occurs immediately after autopolyploidization with no further changes in DNA amount during the life of the autopolyploids or the segmental allopolyploids.

The cytologically diploidized autotetraploid *H. marinum* subsp. *gussoneanum* represents another type of autopolyploids, that is, cytologically diploidized autopolyploids with no reduction in nuclear DNA amount [20]. The nuclear DNA amount of this autotetraploid cytotype found by Vogel et al. [15], Jakob et al. [16], and Eilam et al. [20] did not deviate significantly from its expected additive value. Since no significant changes in DNA amount were found in this tetraploid cytotype, it is assumed that the four partners in each chromosome group have remained homologous. However, this taxon forms exclusively bivalents at meiosis [99]. The bivalents may be formed at random between the four partner homologous chromosomes, that is, different partners in different cells, and, consequently, multisomic inheritance prevails. Alternatively, the bivalents are formed always between the same partner homologues with the resultant disomic inheritance. While multisomic inheritance is characteristic of autohexaploid *Phleum pratense* ($2n = 6x = 42$) which forms 21 bivalents at meiosis but displays hexasomic inheritance [116, 117], and of *Lotus corniculatus* ($2n = 4x = 24$), which forms 12 bivalents at meiosis and has tetrasomic inheritance [118], disomic inheritance,

inferred from isozyme studies, is the mode of inheritance in *H. marinum* subsp. *gussoneanum* [114, 119].

5. Biological Significance of Genome Downsizing in Allopolyploids and Cytologically Diploidized Autopolyploids

Feldman et al. [24], Ozkan et al. [65, 77], Levy and Feldman [120, 121], and Feldman and Levy [25, 26] suggested that rapid and nonrandom elimination of low-copy, noncoding DNA sequences from one of the two homoeologous pairs in tetraploids and from two homoeologous pairs in hexaploids further augments the differentiation of homoeologous chromosomes at the polyploid level and leads to cytological diploidization. Such elimination, which presumably also involved sequences having a role in homology recognition and initiation of meiotic pairing, provides the physical basis for the diploid-like meiotic behavior of allopolyploid *Aegilops* and *Triticum* species. As was found by Ozkan and Feldman [122], the extent of DNA elimination in newly formed allopolyploids was positively correlated with the frequency of bivalent pairing and with seed fertility. Diploid-like meiotic behavior sustains disomic inheritance, which, in turn, maintains favorable intergenomic combinations and permanent heterosis between homoeoalleles. Thus, cytological diploidization might facilitate rapid and successful establishment of the young polyploid in nature, increase its competition with the parental species, and improve its adaptability to new environments.

The low-copy, noncoding sequences that most of which are chromosome specific tend to cluster in several specific regions in each chromosome arm of hexaploid wheat [123]. These regions, being perhaps the only homologous-specific regions in every chromosome pair, may play an important role in processes of pairing initiation at early stages of meiosis [24]. Indeed, recent data (G. Grafi, B. Liu, F. Han, C. Melamed-Bessudo, and M. Feldman, unpublished) indicate that chromosome-specific sequences possess polycomb response elements (PREs) that bind specifically premeiotic proteins. It is speculated (G. Grafi, B. Liu, F. Han, C. Melamed-Bessudo, and M. Feldman, unpublished) that meiotic pairing is initiated by complexing the PREs of the chromosome-specific sequences with premeiotic proteins.

In addition, rapid elimination of low-copy, coding sequences may reduce the dosage of alleles from four to two when extra dose has a negative adaptive value as well as may regulate gene expression, thus leading to improved intergenomic interactions [25, 26]. Rapid nonrandom elimination of repetitious sequences may bring about a greater harmony in the function of the different genomes of the allopolyploid [81]. Likewise, Pai et al. [71] suggested that elimination of chromosomal material from allopolyploid wheat might have been an important factor in the conversion of the allopolyploid species of wheat into functional diploids. Elimination of repetitive sequences may reduce negative nucleotypic effects of increased DNA amount as well as reducing the biochemical costs associated with this extra DNA [10].

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 527357, 4 pages
doi:10.1155/2010/527357

Research Article

New Record Holders for Maximum Genome Size in Eudicots and Monocots

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Received 27 January 2010; Accepted 15 March 2010

Academic Editor: Johann Greilhuber

This paper presents the largest genome sizes reported so far for angiosperms: for the monocots *Trillium hagae* (*Melanthiaceae*) 2C = 264.9 pg and for the eudicots *Viscum album* (Santalaceae) with 2C = 205.8 pg. They were found during ongoing measurements of nuclear DNA content in more than 4000 accessions. Moreover, it is demonstrated that both of the values for the largest genome size so far for the monocots of *Fritillaria assyriaca* and for the eudicots of *Viscum cruciatum* need to be corrected.

1. Introduction

Until recently, genome size in angiosperms varied from 0.321 pg in *Arabidopisis thaliana* (*Brassicacae*) [1] to 254.8 pg in *Fritillaria assyriaca* (*Liliaceae*) [2], an 800-fold range. This genome size is not related to the number of genes (polyploids excluded) but is due to large amounts of repetitive DNA. Also the number of chromosomes is not very relevant as some plants with very high chromosome numbers have low amount of nuclear DNA like *Sedum suaveolens* with about $2n = 640$ and 18.3 pg of DNA (Table 2). This is called the C-value paradox or the C-value enigma [3]. Genome size is not very useful at higher taxonomic levels, but is especially of value at the species level [4–9]. The amount of nuclear DNA is positively correlated with nuclear and cellular volume, whereby mainly cell size is subjected to selection [10].

The smallest angiosperm genomes are found in the *Lentibulariaceae* with *Genlisea margaretae* with a 2C DNA value of only 0.129 pg [11]. This is less than half the size of the genome of *Arabidopsis thaliana* with 2C = 0.321 pg [1] and seems to increase the total range from 800 to 1968-fold. At the other end of the scale always the monocot *Fritillaria assyriaca* with 2C = 254.8 pg is named as the species with the largest genome size for the angiosperms [2]. However, this is based on the wrong assumption [2], as will be demonstrated below, that *Fritillaria assyriaca* has the largest genome size. The supposed largest eudicot is *Viscum cruciatum* with 2C = 158.6 pg. The present study presents the highest values reported so far. They were found during ongoing measurements of nuclear DNA content in more than 4000 accessions [4–9]. They represent a new record at the higher end of the scale of angiosperm C-values. This emphasizes the need for a proper explanation of these very large genome sizes.

2. Material and Methods

2.1. Plant Material. *Trillium hagae* material was obtained from the collection of C. Denton, UK. *Viscum crassulae* was obtained from E. van Jaarsveld of Kirstenbosch Botanical Garden RSA and *Viscum minimum* of C. Grootscholten Succulent Nursery, The Netherlands. *Viscum album* was obtained from the Botanical Garden Wageningen, The Netherlands, and also from Arboretrum Trompenburg Rotterdam, The Netherlands, and collected in south Limburg, The Netherlands; Pyrenees, France, and southern Greece. Live material of *Fritillaria* was contributed by P. Christian, J. de Groot, Hortus Bulborum Limmen, Potterton and Martin, W. Kletzing and M. de Jaeger. Where possible, material of known wild origin was used, and care was taken to ensure correct identification of all materials.

2.2. Flow Cytometric Measurement of Nuclear DNA Content. For the isolation of nuclei, about 1 cm^2 of adult leaf tissue was chopped together with a piece of *Haemantus albiflos* (*Amaryllidaceae)* as an internal standard (see below).

Table 1: Genome sizes (2C) of *Fritillaria* species, standard deviation (SD), chromosome numbers, and number of accessions (#), compared with McLeish and LaCour [1].

Species		2C in pg	S.D.	2*n* *	#	McLeish and LaCour 2C in pg	Difference in pg
Fritillaria	*verticillata*	84.9	1.9	24	4	96.5	11.6
Fritillaria	*pallidiflora*	87.4	1.3	24	2	***116.7	29.3
Fritillaria	*raddeana*	91.1	0.7	24	2	119.5	28.4
Fritillaria	*liliacea*	89.6	1.8	24	1	121.4	31.8
Fritillaria	*pyrenaica*	104.9	3.1	24	3	127.4	22.5
Fritillaria	*meleagris*	112.4	3.0	24	4	141.4	29.0
Fritillaria	*imperialis*	102.5	1.3	24	5	145.0	42.5
Fritillaria	*olivieri*	117.1	0.4	24	3	145.6	28.5
Fritillaria	*acmopetala*	132.5	1.9	24	4	151.1	18.6
Fritillaria	*glauca*	111.7	0.5	24	1	155.3	43.6
Fritillaria	*crassifolia*	133.1	3.5	24	5	160.8	27.7
Fritillaria	*tuntasia*	130.4	1.5	24	2	163.2	32.8
Fritillaria	*pinardii*	131.1	1.6	24	4	164.4	33.3
Fritillaria	*rhodocanakis*	134.9	3.1	24	1	172.3	37.4
Fritillaria	*davisii*	138.9	2.3	24	4	179.0	40.1
Fritillaria	*assyriaca***	203.9	1.4	48	3	254.8	50.9
Fritillaria	*assyriaca*	134.0	4.3	24	4		

* from literature. **= uva-vulpis, [15]. ***Bennett and Smith [16].

The chopping was done with a new razor blade in a Petri dish in 0.25 mL nuclei isolation buffer to which 0.25 mg RNase/mL was added [8]. After adding 1.5 mL propidium iodide (PI) solution (50 mg PI/l in isolation buffer) the suspension with nuclei was filtered through a 30 μm nylon filter. The fluorescence of the nuclei was measured half an hour and one hour after addition of PI, using a Partec CA-II flow cytometer. The optical path contained an HBO mercury lamp, filters KG1, BG12, dichroic mirror TK500, filter OG570, and a Leitz 50 × 1 water immersion objective. Data were analyzed by means of DPAC software (Partec GmbH). If available, three different samples, and at least 3000 nuclei, were measured twice for each clone. Most histograms revealed a Coefficient of Variation (CV) of less than 5%.

2.3. Internal Standard and Absolute DNA Content Values. When measuring nuclear DNA content by means of flow cytometry, it is necessary to chop tissue from the plant of interest together with an internal standard: this standard must be as close as possible to the plants of interest. In this way, variation in signal intensities due to staining kinetics, to light absorption and quenching by sample components, as well as to instrument and other variables, is reduced to a minimum. *Haemanthus albiflos* was chosen as internal standard, because it has a convenient amount of DNA relative to plants with very high DNA values. Moreover, it is available year-round, does not mind several weeks without water, and it can serve numerous determinations. It also has a low background in PI measurements and shows a single G_0 peak, almost lacking G_2 arrest. Fresh male human leucocytes (2C = 7.0 pg [12]) (1 picogram = 0.978×10^9 base pairs [13]) were chosen as primary standard. This yields 2C = 15.9 pg for nuclei of *Agave americana*. This in turn was used to measure *Haemanthus albiflos* and resulted in 2C = 76.0 pg.

3. Results

3.1. The Monocots: Fritillaria, Trillium. The value of 2C = 254.8 pg for *Fritillaria assyriaca* is usually presented as the highest genome size in articles using flow cytometrical methods (this volume!). In the list of plant DNA C-values [2], 19 out of 24 species of *Fritillaria* are by McLeish and LaCour. If these are compared with the present results (Table 1), they are all higher in 2C DNA value with an average increase of 32 pg. It runs from 11 pg higher in *F. verticillata* (96.5 pg,) to 50 pg higher in *F. assyriaca* (254.8 pg). If the present value of 2C = 203.9 pg for *F. assyriaca* hort. (= *F. uva-vulpis*) is used instead of 254.8 pg of McLeish and LaCour, then several other species come in the same league like *F. imperialis* "*Maxima*" with 200.2 pg, and *F. elwesii* with 207.3 pg (Table 1). For real high values one must look to *Trillium* (*Melanthiaceae*). Especially the *trilliums* of Asia have high C-values due to their usually high ploidy [14]. The new record holder presented here is *T. hagae* with 264.9 pg (2*n* = 30). It is an allohexaploid of *T. camchatcense* (2*n* = 10) and *T. tschonoskii* (2*n* = 20). Another high DNA 2C-value is the Asiatic *T. apetalon* (2*n* = 20) with 190.0 pg [5].

3.2. The Eudicots: Viscum. Viscum cruciatum is with 2C = 158.7 pg [17] presented in the list of plant DNA C-values [2] as the eudicot with the largest genome size. For *V. album* a value of 2C = 152.0 pg is chosen [18]. Clearly it is chosen as "prime estimate" as it is intermediate between the value of Nagl et al. for *V. album* with 2C = 107.0 pg [19] and those of Ulrich et al. with 2C = 181.5 pg [17]. It would have been better to choose both values from a single laboratory. Ulrich et al. [17] found the 2C-value of *V. album* to be higher than those of *V. cruciatum* and that is confirmed here. Ulrich et al.

Table 2: Comparison of genome sizes (2C in pg) in this paper. # = number of accessions.

Species		2C in pg	S.D.	2*n* *	#	Nagl et al. [19]	Marie and Brown [18]	Ulrich et al. [17]
Trillium	*hagae*	264.9	3.2	30	1			
Trillium	*apetalon*	190.0	4.1	20	2			
Viscum	*album*	205.8	3.9	20	6	107.0	**152.0	181.6
Viscum	*cruciatum*	175.8	2.6	20	2			**158.7
Viscum	*crassulae*	164.0	3.6	24	1			
Viscum	*minimum*	124.6	3.2	28	2			
Fritillaria	*elwesii*	207.3	2.3	48	3			
Fritillaria	*imp.Maxima*	200.2	0.3	48	2			
Fritillaria	*assyriaca*	134.0	4.3	24	4			
Haemanthus	*albiflos*	76.0	2.1	32	6			
Loranthus	*europaeus* [20]	16.5						
Orobanche	*hederae* [4]	5.5	0.4	38	2			
Sedum	*suaveolens*	18.3	2.4	c640	4			

* from literature. **"Prime values" [2].

used several both A-T and G-C specific dyes and from these results calculated the "absolute DNA content." They also used Feulgen for *V. album* and obtained nearly the same result (184.4 pg). The present results for these species are 2C = 205.8 pg for *V. album* (2*n* = 20), and 2C = 175.8 pg for *V. cruciatum* (2*n* = 20) (Table 2). Also *V. crassulae* with 2C = 164.0 pg (2*n* = 24) and *V. minimum* with 2C = 124.6 pg (2*n* = 28) are here presented as new DNA 2C-values (Table 2).

4. Discussion

Most of the values of *Fritillaria* in the list of plant DNA C-values are from McLeish and LaCour from 1971 [2]. They were measured with Feulgen cytometry and based on a personal communication. The standard species is unknown and they might not have used "best practice" as advocated for the Feulgen method [20]. The increasing difference of 11–50 pg going from the lowest to the highest value in *Fritillaria* as presented by McLeish and LaCour compared with the present results (Table 1) suggest problems with the linearity of their measurements. Actually a 2C DNA value of 134 pg was found for four accessions of the diploid *F. assyriaca* (Table 1). It cannot be excluded that McLeish and LaCour had a tetraploid *F. assyriaca.* However, it is more likely, they used the plant that is in the trade as *F. assyriaca,* but actually is the tetraploid *F. uva-vulpis* with 2C = 203.9 pg. Moreover, Rix (1974) [15] has shown that up to that time all plants in commerce as *F. assyriaca* were a separate species *F. uva-vulpis*. The high values of McLeish and LaCour can be explained by the fact that they were obtained after recalibrating their values against *F. pallidiflora* with 116.7 pg [16]. The present results show *F. pallidiflora* to have only 87.4 pg (Table 1).

The combined results of the 2C DNA values of 0.129 pg for *Genlisea margaretae* [11] with those presented here with 264.9 pg for *Trillium hagae* increase the range of genome sizes for angiosperms to 2056-fold.

It may be wondered whether the high value found for *V. album* has anything to do with its parasitic nature. This seems not likely as the related tree parasite *Loranthus europaeus* has 16.5 pg [18] and *Orobanche hederae* has 5.5 pg [5]. The large genome size seems also not an impediment to growth rate. *V. album* on an apple tree in my garden reached football size and bear fruits in three years from seed.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 504613, 8 pages
doi:10.1155/2010/504613

Research Article

Comparative Analysis of Growth, Genome Size, Chromosome Numbers and Phylogeny of *Arabidopsis thaliana* and Three Cooccurring Species of the Brassicaceae from Uzbekistan

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Received 1 December 2009; Accepted 25 February 2010

Academic Editor: Johann Greilhuber

Contrary to literature data *Arabidopsis thaliana* was rarely observed in Middle Asia during a collection trip in 2001. Instead, three other Brassicaceae species were frequently found at places where *A. thaliana* was expected. To reveal reasons for this frequency pattern, we studied chromosome numbers, genome sizes, phylogenetic relationships, developmental rates, and reproductive success of *A. thaliana, Olimarabidopsis pumila, Arabis montbretiana*, and *Arabis auriculata* from Uzbekistan in two temperature treatments. There are little but partially significant differences between phenotypes. All studied species have very small genomes. The 1Cx-values of different genotypes within the sampled species are correlated with altitude. Developmental rates are also correlated with 1Cx-values. In our growth experiments, *Arabidopsis* had high seed sterility at higher temperature, which might be one reason for the rarity of *A. thaliana* in Middle Asia.

1. Introduction

Arabidopsis thaliana is a model plant in many aspects of plant biology. Naturally occurring variability of the species may be a valuable source for biochemical and genetic analyses (reviewed in [1, 2]). Usually, *A. thaliana* is investigated separately or with congeners in studies dealing with ecologically relevant characters. Here, we compare *A. thaliana* with related species of other genera growing in the same or similar ecological niche in Middle Asia, particularly Uzbekistan. The starting point for this comparative analysis were observations on an expedition to Uzbekistan in spring 2001 where we studied *A. thaliana* in the field at its eastern distribution boundary [3]. Literature data suggested *A. thaliana* to be quite common in different types of habitat and occurring in wide altitudinal ranges of Middle Asian mountains (e.g., [4–7]). We also studied all available vouchers in the herbarium in Tashkent (TASH, Uzbekistan) for localities where the species were collected and for detailed ecological data. *Arabidopsis thaliana* was, in contrast to literature data, very rare or absent in the field in comparison to three other annual species of the Brassicaceae growing at same places or observed in the following years at these places (R. Fritsch, pers. obs.). The three other species investigated *Olimarabidopsis pumila*, *Arabis montbretiana,* and *Arabis auriculata* are of similar growth, vegetative structure, and ecology and were found in high abundances at places where we expected *A. thaliana.* The reasons for the absence of *A. thaliana* in 2001 were not obvious in the field, but we suspect that climate in conjunction with morphological and DNA 2C-value may provide an explanation for this observation.

Arabidopsis thaliana, *O. pumila*, *Arabis montbretiana*, and *A. auriculata* are annuals having distinct rosettes of leaves

but also several cauline leaves. The fruit of all taxa are siliques. *Olimarabidopsis pumila* (syn. *Arabidopsis pumila*) is a yellow flowering species mostly observed in the lowlands and on riverbanks. The two white flowering *Arabis* species have a tendency to occur more frequently in foothills and mountains. Rather contradictory taxonomic treatments of the species can be found in older literature. Using molecular analysis of the ribosomal internal transcribed spacer (ITS) sequences, the distinctness of the species became apparent. Sometimes the species intermingle locally but are readily distinguishable from *A. thaliana*, which has no clasping cauline leaves. All taxa are selfing, and interspecific hybridisation is hardly possible (e.g., [8]).

The aim of this study is to compare the four species with respect to chromosome numbers and genome sizes in relation to their phenological behaviour under controlled conditions. Growth chamber conditions that simulate constraints and competition of the plants in natural habitats are difficult to design. Temperature is considered to be one of the major environmental factors influencing plant distribution (e.g., [9]). Therefore, we decided to study the developmental rates and the reproductive success of the four species in two controlled temperature conditions, that is, 14°C and 22°C. The following questions will be addressed by our experiments: (1) are there different developmental rates of the co-occurring plant species in two different temperature treatments and does temperature significantly favour or retard species? (2) How do the developmental rates and responses correlate with the chromosome numbers and genome sizes at different temperatures?

2. Materials and Methods

2.1. Molecular Phylogenetics. The following taxa were included in our analysis (species name and GenBank accession numbers are given, resp.): *Aethionema arabicum* (AY254539), *Arabidopsis thaliana* (AJ232900), *A. cebennensis* (AF137545), *A. lyrata* (AJ232889), *A. lyrata* ssp. *petraea* (AJ232891), *A. lyrata* ssp. *kamchatica* (U96266), *A. croatica* (AF137546), *A. neglecta* (U52186), *A. arenosa* (U52188), *A. halleri* ssp. *gemmifera* (AF137544), *A. halleri* ssp. *halleri* (AF137541), *Olimarabidopsis korshinskyi* (AJ232931), *O. pumila* (AF137549), *O. griffithiana* (AJ232911), *Capsella bursa-pastoris* (AF055196), *Crucihimalaya wallichii* (AJ-131396), *Arabis pendula* (AF137572), *A. lignifera* (AJ-232899), *A. holboellii* (AY457932), *A. lyallii* (AF137561), *A. drummondii* (AF137575), *A. parshii* (AJ232902), *A. turrita* (AJ232906), *A. hirsuta* (AJ232886), *A. procurrens* (AJ232917), *A. nuttallii* (AF137562), *A. flagellosa* (AF-137560), and *A. alpina* (AF137559).

The following taxa were sequenced (the acronym HAL refers to vouchers in the herbaria of Halle, Germany, GAT for Gatersleben, Germany): *A. auriculata*: Austria, Bruck, Niederdonau (HAL 30014); Spain, Burgos (HAL 84268); Bulgaria, Golo Bardo (HAL 72898); Germany: Kyffhäuser (GAT 6359). DNA isolation and sequencing of the ribosomal ITS region were done as described in [10]. The plants of *A. auriculata* and *A. montbretiana* from Uzbekistan were grown from collected seed material in the greenhouse in Gatersleben.

The analysis was run using PAUP 4.0* beta version [11] following the settings by Koch et al. [12]: HEURISTIC, TBR, STEEPEST DESCENT. Insertions and deletions were treated as one event each. Characters and character states were weighted equally (Fitch parsimony). The bootstrap option of PAUP 4.0* (1000 replicates) was used to assess relative support. An estimate of the phylogenetic signal present within the ITS data was obtained using the Random Trees option. One thousand bootstrap samples were analysed to assess the significance of nodes on the original neighbour joining tree.

2.2. Flow Cytometry. For preparation of suspensions of nuclei, about 30 mg of fresh leaf tissue was chopped with a razor blade together with material from the reference plant in 1 ml ice-cold staining buffer in a Petri dish and analysed according to the protocol of Barow and Meister [13]. A FACStar^{PLUS} flow cytometer (Becton Dickinson, San José, CA, USA) equipped with two argon lasers INNOVA 90-5 (Coherent, Palo Alto, CA, USA) was used (excitation at 514 nm, emission at 630 nm). The data were analysed with the program CellQuest (Becton Dickinson). DNA content of the nuclei was estimated by the fluorescence of the nuclei of samples stained with propidium iodide relative to the internal standards *Raphanus sativus* (2C = 1.10 pg, for *Arabidopsis thaliana)* and *Glycine max* (2C = 2.25 pg, for the other species) [14]. Usually 10,000 nuclei were measured.

2.3. Chromosome Counting. Root tips (from plants grown in pots or from seedlings) were treated for 45 minutes with saturated paradichlorobenzene solution in water and were fixed in a mixture of methanol and formic acid (vols. 3 : 1) under room temperature overnight. After maceration in n-HCl (7 minutes, 37°C) root tips were squashed in a drop of acetic acid (45 vol. %), and chromosomes were analysed under a light microscope using phase contrast.

2.4. Growth Chamber Experiment. Single seeds of each species' original collection in Uzbekistan (Table 1) were placed in 6.5 × 6.5 cm pots filled with the autoclaved Klassmann Substrat2 substrate (Klassmann-Deilmann GmbH, Geeste, Germany), moistened and stratified in the dark at 4°C for 7 days. Thereafter, the plants were transferred to growth chambers at constant 14°C and 22°C, respectively. Long-day conditions (20 hours light, 4 hours dark) were maintained during the experiment. In total, up to five plants per accession were cultivated for *A. montbretiana*, *A. Auriculata,* and *O. pumila.* For *A. thaliana* more plants were grown. In order to minimise microenvironmental effects, trays containing the pots were randomly rearranged on the shelves every second day. Pots were not allowed to dry out.

The following phenological characters (phenological stages) were recorded: days until germination, days until bolting, days until opening of the first flower, and days until opening of the first fruit (siliqua).

Table 1: Overview of Uzbek species grown in the experiment including geographical coordinates and altitudes. Grouping of genome size values within the species based on the Tukey test is indicated by superscript letters. Accessions with the same letter are *not significantly different at 5% level.*

Location	North	East	Altitude	DNA 2C-values (pg DNA) Mean	SD	*N*	Chromosome number (2n)	1Cx DNA amount (Mbp)
Olimarabidopsis pumila								
Buchara	39°48′	64°25′	225 m	0.708[a]	0.015	4	32	173
Jizzakh	40°10′	67°40′	500 m	0.737[b]	0.007	4	32	180
Mirzaaul	40°38′	68°47′	400 m	0.755[bc]	0.018	4	32	185
Shur-Ob	38°12′	66°58′	1100 m	0.772[c]	0.010	4	32	189
Arabidopsis thaliana								
Sidzhak	41°44′	70°05′	1100 m	0.342	0.004	10	10	167
Arabis auriculata								
Khumsan	41°41′	69°57′	850 m	0.451[a]	0.008	4	16	221
Sidzhak	41°44′	70°05′	1100 m	0.459[a]	0.008	4	16	224
Arabis montbretiana								
Galabasaj	41°33′	69°52′	1100 m	0.562[a]	0.001	4	16	275
Belder-Saj	41°30′	69°55′	1100 m	0.568[ab]	0.008	4	16	278
Sukok	41°15′	69°49′	1500 m	0.575[ab]	0.004	4	16	281
Aksakata-Saj	41°23′	69°54′	1100 m	0.576[ab]	0.005	4	16	282
Sangardak	38°33′	67°33′	1500 m	0.579[bc]	0.010	4	16	283
Aman-Kutan	39°17′	66°54′	1650 m	0.596[cd]	0.008	4	16	291
Chetsuv	41°12′	70°15′	1800 m	0.601[d]	0.011	4	16	294

The proportion of ripe seeds per locus was calculated using the formula: (number of ripe seeds − number of sterile ovules)/(total number of ovules). The result of the formula is between +1, that is, all ovules developed into ripe seeds, and −1 indicating that all ovules are sterile. Due to this procedure the relative reproductive success of the different species in the treatments can be compared.

2.5. Statistics. Statistics were calculated using the program SPSS (SPSS for Windows. 1999. Chicago: SPSS Inc.). After testing for normality, analysis of variance (ANOVA) was performed. Tukey post hoc tests were calculated to find significantly different groups of species or accessions.

3. Results

3.1. Taxonomy, Chromosome Numbers, Genome Size, and Molecular Analyses. The taxonomy of the two *Arabis* species included in the analysis caused several problems. The plants collected in Uzbekistan were identified as *A. montbretiana* and *A. auriculata*, respectively [6]. Titz [15] considers both taxa to be conspecific and lists *A. montbretiana* as a synonym of *A. auriculata*, which is a very widespread taxon in his treatment ranging from Europe to Middle Asia. The Middle Asian *A. montbretiana* appears, however, to be a species rather well distinguished from *A. auriculata* in having seeds >1 mm and leaves with rounded and coarse lobes. *Arabis auriculata* has small seeds (<1 mm long) and more or less entire leaves with a partly dentate margin.

Despite that Flora Europaea [16] considers *A. auriculata* Lam. (1783) to be a synonym of the widespread taxon *A. recta* Vill, (1788) the nomenclature of *A. auriculata* is unambiguous. The plants are very similar having a sepal length of less than 4 mm, fruit pedicel of <4 mm and a fruit diameter of <1 mm. They can be distinguished from *A. nova* Vill., which is larger in all parts.

Chromosome numbers were counted for plants collected in Uzbekistan (Table 1). *Olimarabidopsis pumila* was confirmed to have $2n = 32$ chromosomes, the two *Arabis* species have $2n = 16$ chromosomes each. Determination of chromosome morphology was difficult because of the small size between 0.5 and 1.6 μm length and less than 0.3 μm width. Only very few well spread metaphase plates showed centromeres but were not sufficient for statistical analyses of karyotypes. Both *Arabis* species shared two long chromosomes having possibly satellites. Six medium-sized and four short chromosomes were apparently metacentric. No conspicuous karyotype differences were found between these taxa. Most chromosomes of *Olimarabidopsis* were somewhat smaller, but the length of one pair reaches nearly 2 μm. The other chromosomes differed little in length showing metacentric and sub-metacentric centromere positions. These data widely agree with the figure of a metaphase plate given by Manton [17].

2C-values [18] were determined (Table 1) revealing a twofold difference among the species. *Arabidopsis thaliana* has the smallest genome [19] followed by *A. auriculata* and *A. montbretiana*. Mean 2C-values of species are highly correlated with chromosome numbers ($n = 4$, Spearman's

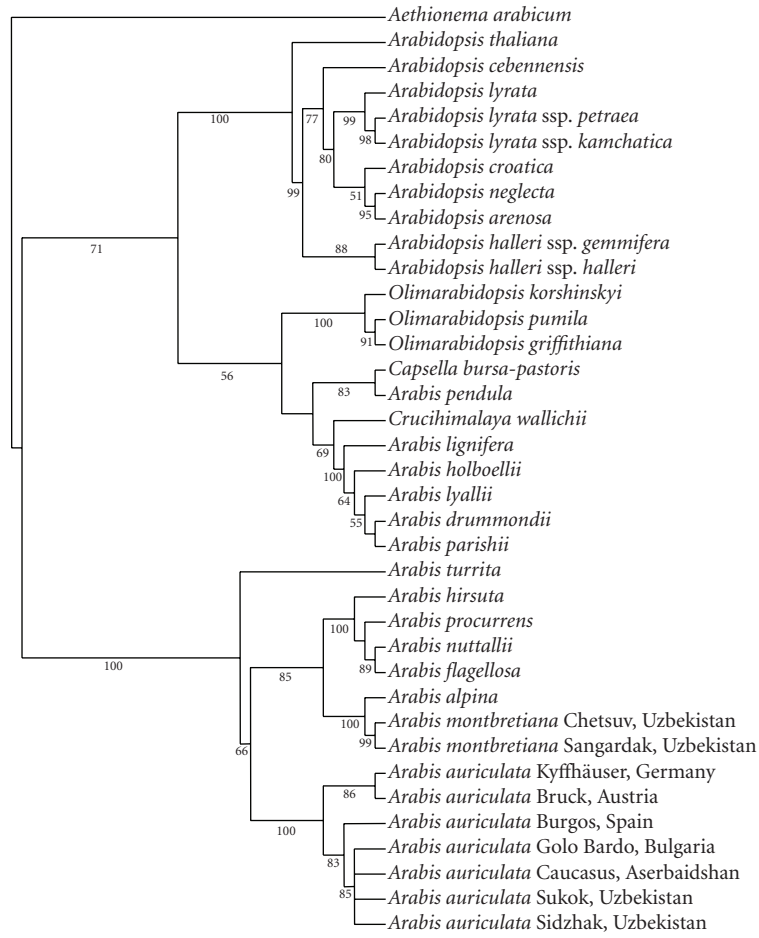

Figure 1: Strict consensus tree of 15 most parsimonious trees from the ribosomal ITS data set. Bootstrap values in percent are indicated below the branches. The *Boechera*-clade comprises, for example, *A. lyallii*. The *Arabis*-clade is the large clade at the bottom of the cladogram.

correlation coefficient = 0.949). However, this correlation is not significant at the 0.05 level ($P = .0833$) because of the small number of species. The *Arabis* species are well distinguished by their different 2C-values. The largest genome was observed in *O. pumila*, which is about twice as large as that of *A. thaliana*. We observed a number of significant differences of 2C-values among the populations of *A. montbretiana* and *O. pumila*. (Table 1) despite the rather low sample size used for comparison. The same was observed for *A. thaliana* [19]. The two populations of *A. auriculata* do not significantly differ in their 2C-values. A significant positive correlation between 1C-value and altitude was found within the species *O. pumila* ($r = 0.728$, $P = .0011$) and *A. montbretiana* ($r = 0.803$, $P < .001$). *A. auriculata* gives also a clear correlation ($r = 0.546$), but because of the low amount of data (only 2 different altitudes) it is not significant ($P = .139$). For *A. thaliana,* correlation cannot be calculated because all plants are collected at the same altitude.

The phylogenetic tree of the ribosomal ITS data (strict consensus tree of 15 maximum parsimony trees) is given in Figure 1. The tree topology is similar to the ITS data set of Koch et al. [12] and the combined data set of the *matK-Chs* data set [20], particularly for *Arabidopsis* and *Olimarabidopsis*. *Arabis* is a polyphyletic genus in these trees. One clade is named *Boechera* and the other *Arabis*. The two *Arabis* species included in our data set group together with other *Arabis* species not included in *Boechera*. *Arabis montbretiana* is most closely related to *A. alpina* and belongs to a larger clade that is a sister group to a clade comprising *A. auriculata*.

Three subclades with considerable bootstrap support can be distinguished within the *A. auriculata* clade. The two plants from the Kyffhäuser mountains (Germany) and from Bruck (Austria), respectively, are basal in this clade. Finally, a plant from Burgos (Spain) is basal to a group of plants from south-eastern Europe (Bulgaria), the Caucasus (Azerbaidzhan), and Middle Asia (Uzbekistan).

3.2. Growth Chamber Experiments. The species performed unequally well in the growth chambers experiments. Olimarabidopsis, *A. thaliana,* and *A. montbretiana* grew almost without problems. *Arabis auriculata* performed less well, that is, some plants died at several stages of the development. Maybe some cold period was missing for a proper development in our controlled environment experiment.

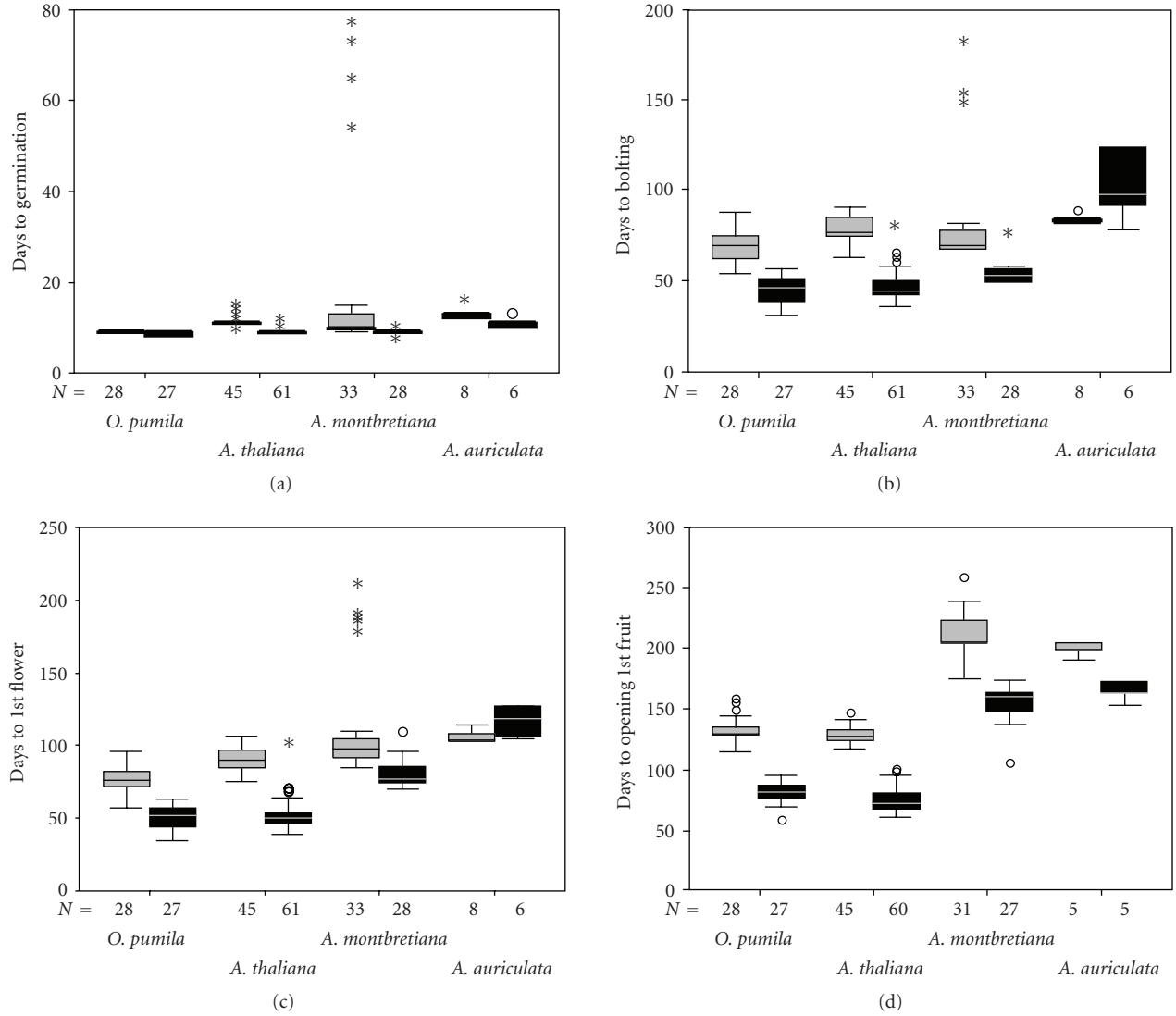

Figure 2: Boxplots showing the phenological stages of the studied species. The two treatments (14 and 22°C) are plotted together: the grey boxes demarcating the 25th and 75th percentile (interquartile range), with the black median line referring to the 14°C treatment, the black boxes with the grey median to the 22°C treatment. The error bars mark the largest and smallest value, respectively. An open circle marks outliers, extreme values are marked by an asterisk. The number of studied plants is indicated below the columns. The number may decline from one stage to another due to death of plants.

Analysis of variance (ANOVA) revealed significant differences between the developmental rates among the species within each of the treatments (data not shown). These differences may also be inferred from the boxplots shown in Figure 2. However, some species did not differ significantly in their phenological phases. Groups of species not significantly differing from one another are shown in Table 2.

The numbers of days until germination (Figure 2(a)) are comparable among the species in the treatments. *Arabis auriculata* and *A. thaliana* did not differ significantly between the treatments. In the 22°C treatment all taxa germinated earlier than in the 14°C treatment. *Arabis montbretiana* revealed a remarkable pattern that consisted apparently of two different plant types: one with early and one with late germination. These late germinating seeds were collected among early germinating seeds in Sukok and Chetsuv. The reason for this difference is unknown. It could be either different genotypes growing together in the wild or perhaps epigenetic modifications that regulate dormancy. These germination types were not visible in other species of the 14°C experiment. In the 22°C treatment these differences were not observed.

The numbers of days until bolting (Figure 2(b)) were similar between the species in the 14°C treatment; the fastest developing species were *O. pumila* and *A. montbretiana*. All species bolt earlier in the 22°C treatment with the exception of *A. auriculata*. *Arabidopsis thaliana* got the strongest growth acceleration in higher temperatures. It needed the same time to this phenological stage as *O. pumila*. In the following stage, that is, the opening of the first flower

Table 2: Groups of similarly developing species in the 14°C (above diagonal) and 22°C (below diagonal) treatment as revealed from ANOVA and subsequent Tukey's significant difference test. For example, in the 22°C treatment *A. thaliana* and *O. pumila* developed similarly in the phenological stages: B-days until bolting, F-days until flowering, and Fr-days until opening of the first fruit. Further abbreviation: G-days until germination. Nonoverlapping groups of species from the Tukey's test are marked in bold italics letters. For example in the 14°C treatment, *O. pumila* and *A. thaliana* are not significantly different from one another in the days to opening of the first fruit, but they differ significantly from the *Arabis* species that are not significantly different from one another.

	A. thaliana	*O. pumila*	*A. montbretiana*	*A. auriculata*
A. thaliana	—	G, B, Fr	B	G, B, F
O. pumila	B, F, Fr	—		G, B
A. montbretiana			—	G, B, F, Fr
A. auriculata				—

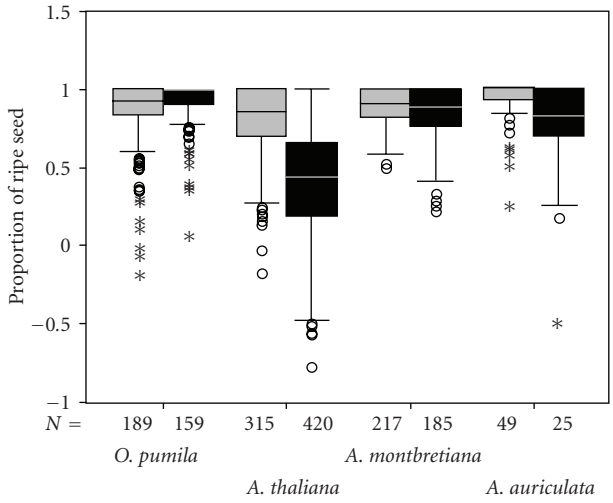

Figure 3: Boxplots showing the proportion of developed seeds to the total number of ovules. For further explanation see Figure 2.

(Figure 2(c)), *A. montbretiana* developed slower in the 14°C treatment than *O. pumila*. In the 22°C treatment *O. pumila* and *A. thaliana* did not significantly differ from one another and developed faster than *Arabis*. *Arabis auriculata* had a still slower development at higher temperatures.

The seeds of *A. thaliana* and *O. pumila* ripened very rapidly (number of days until opening of the first fruit, Figure 2(d)). The fruit of *A. auriculata* developed also very fast, especially in the 22°C treatment where the above-mentioned pattern of slow development was reversed. The late germinating plants of *A. montbretiana* were still delayed to the bulk of the other species but not as strong as during the early phenological stages.

The proportion of ripe seeds was significantly different among all species and treatments (Figure 3). In the 22°C treatment *O. pumila* produced a higher proportion of ripe seeds than the other species. Most apparent was the pattern of higher seed sterility in *A. thaliana*, which produced considerably less ripe seeds in the 22°C treatment.

Correlations between the 2C-value genome sizes of the species and developmental rates were statistically not significant (data not shown). Correlating the 1Cx-value with the developmental rates of the two growth conditions yielded

Table 3: Correlations between 1Cx-value of the species and developmental rates, that is, days until germination, bolting, flowering, and fruiting. Spearman correlation coefficients are given for the two treatments ($n = 14$). Asterisks indicate significant correlations ($P < .05$) after treatment-wise sequential Bonferroni correction.

	14°C	22°C
Germination	0.534	0.052
Bolting	0.218	0.622
Flower	0.631*	0.579
Fruit	0.789*	0.656*

significant results (Table 3). In both treatments the days until opening of the first fruit were significantly correlated with the 1Cx-value. The two *Arabis* species having larger 1Cx-values developed slower than the two other species.

4. Discussion

The cruciferous species co-occurring with *A. thaliana* in Uzbekistan belong to different tribes of the Brassicaceae. The taxonomy of *Arabis auriculata* raises some interesting issues. Although morphologically similar, the individuals of *A. auriculata* from Europe and Middle Asia are distantly related from one another and form sister clades in our phylogenetic analysis with high bootstrap support. Either *A. auriculata* may comprise several cryptic species (e.g., reviewed by Brochmann and Brysting [21]) or is one quite polymorphic species. If *A. auriculata* turns out to be polymorphic, the basal clade comprises plants from northern areas of Europe, while the plants of the sister clade are from the Mediterranean region, the Caucasus, and Middle Asia. This might point to a migration of the plants from Europe to Middle Asia. Taking the scenario of cryptic species, the Southern taxa may have diverged from a taxon distributed in the North. *Arabis montbretiana* is in our analysis a sister clade to *A. alpina*, a rather widespread species of the northern hemisphere having isolated occurrences also in the East African mountains.

The species included in our analysis differ largely in genome size and chromosome numbers, but still belong to plant species having very small genome sizes. Genome size is potentially tied to all features of cell division rates,

for example, the growth rates of plants [22–24]. Perennial plant species with larger genomes tend to have higher rates of shoot growth in cold conditions [23, 25]. Reduction in genome size in maize is correlated to earliness, that is, a fast development and finishing of the life cycle of the plants [26]. Similarly, a reduction in genome size is apparently a very important prerequisite for the evolution of annuals [27]. We observed remarkable differences in chromosome numbers and genome sizes and could relate these differences to developmental rates in the experiments. The species with the smallest genome (2C-value and 1Cx DNA amount) of our experiment (*A. thaliana*) develops in many phenological phases and stages similarly fast as *O. pumila* that has the largest 2C-value but a comparable 1Cx-value (Table 1). The *Arabis* species have intermediate 2C-values but higher 1Cx-values and develop almost always slower than the other two species. The 1Cx-value of the four species may thus explain phenological differences and similarities among the species at least for the later developmental stages. Chromosome numbers are not correlated with the developmental rates of the plants. The average DNA content per chromosome only partly correlates with developmental rates. *Olimarabidopsis pumila* has the lowest mean DNA content per chromosome that is followed by *A. auriculata* and the two remaining species. Considering the studied taxa in their phylogenetic context, that is, clade-wise, a partial compensation of the genome size by the chromosome number can be observed in the clade comprising *A. thaliana* and *O. pumila*. The more-than-threefold higher number of chromosomes in *O. pumila* compared to *A. thaliana* compensates partly the about twofold higher 2C-value of *O. pumila*. Thus, the 1Cx-value is approximately the same between the species. In the other clade including the *Arabis* species the mean DNA content per chromosome correlates with the genome size because the taxa have the same chromosome numbers.

To reveal reasons for the rarity of *A. thaliana* in Uzbekistan, we chose temperature as one of the major environmental factors to study the performance of co-occurring plants of apparently similar ecology and life pattern. Increasing temperature has a differential influence on the growth rates of plants. Morse and Bazzaz [28] observed a higher initial growth of plants due to higher temperatures but also an increased competition among the plants. Dunnett and Grime [25] made a similar observation that increasing temperatures had positive effects on the development of plant monocultures. However, in mixed cultures divergent responses of the species were observed. The plants studied in our experiment were found growing together in Middle Asia where climate for annual plants is most favourable from autumn to spring with a short disruption in winter. In general, the growing season is distinctly shorter than in northern area due to the rapid onset of the hot and dry summer. *Olimarabidopsis pumila* appears to be the species best adapted to a short growing season. This species develops very fast and has a high fertility in higher growing temperatures. *Arabidopsis thaliana* has a similarly fast development but at 22°C seed sterility increased. Increasing pollen sterility at higher temperatures may be the reason for an enhanced abortion of ovules [29]. Other reasons for a higher abortion rate may be developmental defects during the maturation of the seeds as observed in *Brassica* [30]. Temperature may, therefore, be a principal factor for the rarity or absence of *A. thaliana* from the hot and only shortly moist Middle Asian lowlands [3], where temperatures increase very rapidly in spring. The high temperatures might favour a rapid development of the species but the increasing seed sterility may exterminate the species over many generations. In this respect, *O. pumila* may be better adapted. However, *A. thaliana* may produce a soil seed bank [31, 32] from which plants can be recruited in favourable years with cooler springs.

The two species of *Arabis* develop significantly slower in most cases than the two above-mentioned species. However, their abundance in Uzbekistan point to the fact that they are also well adapted. We observed a distinct allocation of nutrients into root biomass in the experiment, that is, much more roots appeared at the bottom of the pots than in the other species. It might be possible that the roots of these species reach deeper and thus, in moister soil layers. Consequently, the slow development might not be a disadvantage because the plants can use a longer vegetation period than the other species having only a shallow root system. The differences between the two *Arabis* species are not very pronounced, which is in accordance with their frequent joint natural occurrence. However, for unknown reasons *A. auriculata* developed less well in the experiment as *A. montbretiana.*

Our experiments revealed distinct differences in the developmental rates of the species and their fertility in dependence of temperature. Although significantly different in our experiments the plants grow together in the field. The different response of the species to temperature, particularly observed in the vegetative development of the *Arabis* species and the seed set of *A. thaliana,* suggests that the species may be favoured differently in annually fluctuating weather conditions. This may balance their cooccurrence. It may be possible that instead of temperature the amount of precipitation may have been the reason for the observed rarity of *A. thaliana* in Uzbekistan in spring 2001. This would be in accordance with observations of Zavaleta et al. [33] that warming did not alter the species composition in a California annual grassland community but precipitation. Further studies are necessary to solve this question.

In our species set we observed correlations of 1Cx-values with some phenological characters as well as the altitudinal distribution of the species. Low 1Cx-values were observed in species of the dry and hot lowlands of Uzbekistan and species of the more mesic mountain habitats had higher 1Cx-values. This pattern may be in accordance with general observations that species with lower 2C DNA-values may have a wider distribution with respect to temperature and precipitation than those with high values [34].

Acknowledgments

The authors thank K. Bachmann for discussions. The technical assistance of I. Faustmann and B. Sperling is

gratefully acknowledged. We thank also to F. Khassanov and the Botanical Institute of the Uzbek Academy of Sciences, Tashkent, for scientific assistance and technical support during the field work in Uzbekistan. The work was supported by a grant of the German Science Foundation (DFG).

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 612017, 7 pages
doi:10.1155/2010/612017

Research Article

On the Relationship between Pollen Size and Genome Size

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Received 22 December 2009; Accepted 2 April 2010

Academic Editor: Ilia Judith Leitch

Here we test whether genome size is a predictor of pollen size. If it were, inferences of ancient genome size would be possible using the abundant paleo-palynolgical record. We performed regression analyses across 464 species of pollen width and genome size. We found a significant positive trend. However, regression analysis using phylogentically independent contrasts did not support the correlated evolution of these traits. Instead, a large split between angiosperms and gymnosperms for both pollen width and genome size was revealed. Sister taxa were not more likely to show a positive contrast when compared to deeper nodes. However, significantly more congeneric species had a positive trend than expected by chance. These results may reflect the strong selection pressure for pollen to be small. Also, because pollen grains are not metabolically active when measured, their biology is different than other cells which have been shown to be strongly related to genome size, such as guard cells. Our findings contrast with previously published research. It was our hope that pollen size could be used as a proxy for inferring the genome size of ancient species. However, our results suggest pollen is not a good candidate for such endeavors.

1. Introduction

Pollen range in size by over three orders of magnitude [1, 2] (Figure 1). The variation in pollen size may stem from strong selection pressures related to pollen dispersal strategies. For example, wind-pollinated species may achieve long-distance transport by having pollen that are (1) small, (2) light weighed, (3) dehydrated, and (4) that have shapes conducive to wind capture [1–3]. However, some gymnosperms have large pollen but are also wind pollinated (Pinaceae and Podocarpaceae) [4]. Two air-filled sacs (sacci) facilitate wind dispersal in these groups [3, 4]. Pollen of species that use insect facilitated dispersal can sometimes be quite large, but we are not aware of any study showing that pollen dispersed by insects is generally larger than pollen dispersed abiotically. However, there is greater interspecific variability for pollen grain size in species that use insect dispersal [5–7]. Understanding what controls pollen size from a developmental perspective will enhance our understanding of the ecological significance of variation in pollen size.

It has frequently been observed that pollen size is related to the length of the style (see [8, 9] and citiations therein). Delpino [8] suggested that larger pollen grains contain more resources for the growth of pollen tubes and therefore larger pollen is better suited to fertilize flowers with longer styles. Darwin [10] disagreed with this proposal, suggesting that pollen tube growth was facilitated by resources garnered from the style. Closely related species sometimes exhibit extreme variation in pollen size and style length. A change in style length may ensure reproductive isolation, especially if style length increases with pollen size, and larger pollen may be necessary for pollination of flowers with longer styles [8, 9]. Conversely, there may simply be inherent allometric determinants of organ size that are shared between both pollen, styles, and other plant parts (see [11, 12] for a current review of genetic determinants of organ size).

Recently, Beaulieu et al. [13] found a strong positive relationship between genome size and cell size, leaving open the possibility that genome size may partly determine, or be correlated with pollen size. A pollen grain consists of

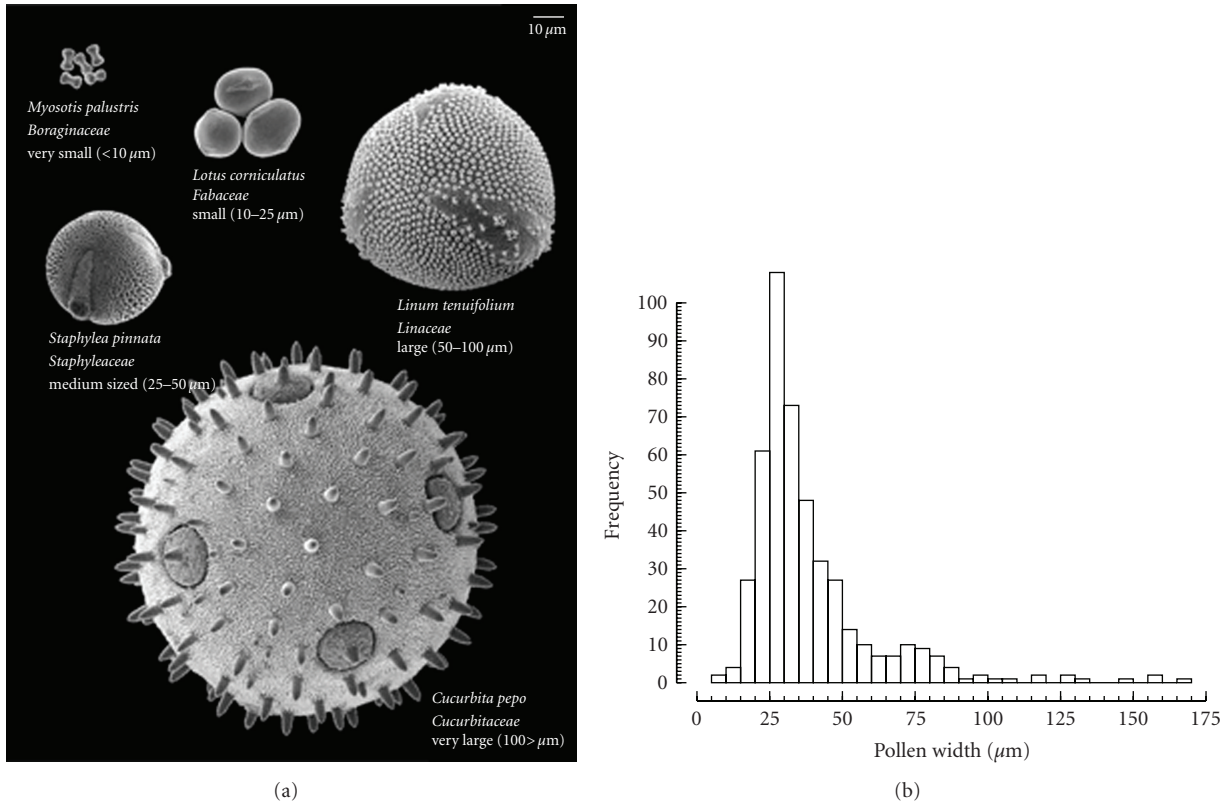

Figure 1: Pollen varies considerably in size. (a) Images of pollen at the same scale and (b) (Inset on a): a histogram of pollen widths showing a log normal distribution.

a vegetative cell and a generative cell. The generative cell is enclosed within the cytoplasm of the vegetative cell. For our purposes, we refer to pollen as unicellular, yet it is clear that the cellular composition of the vegetative cell is unique. Previous reports suggested that pollen size increases with ploidy [14–17]. For example, Bennett [17] found that pollen size increased in proportion to genome size in 16 grass species. If there is a strong association between pollen volume and genome size, it might be possible to infer genome sizes and/or ploidy for species in the fossil record.

Here we perform a large scale analysis of the relationship between pollen size and genome size encompassing 464 species (437 angiosperms and 27 gymnosperms). We assembled pollen size information (equatorial diameters, see methods for more complete description) from the primary literature and from our own measurements and matched these values with the Plant DNA *C*-values database [18]. Here we define genome size as the nuclear DNA content of the unreplicated gametic genome (the monoploid genome size sensu [19]). We also assembled published reports on the relationship between ploidy levels and pollen size.

2. Methods

Estimates of DNA content were compiled from the Plant DNA *C*-values database maintained at the Royal Botanical Gardens, Kew [18]. Equatorial diameters for spheroidal (or near spheroidal) pollen were compiled from various sources including: (1) The Northwest European Pollen Flora periodically monographed by family in the Review of Palaeobotany and Palynology (114 species) [20–23] and others, (2) the palynological database (http://www.paldat.org/) an online publication of the Society for the Promotion of Palynological Research in Austria (122 species), (3) direct measurements by Leighton Dann using light microscopy (157 species—water suspension), and (4) various primary literature sources (71 species). For gymnosperms equitorial diameters only included the central spehere, not the peripheral structures. All values of genome size and pollen width are listed in our supplementary table mentioned in SM available online at doi: 10.1155/2010/612017.

We used Phylomatic (tree version: R20080417.new, maintained by C. A. Webb, http://www.phylodiversity.net/phylomatic) to construct a "mega-tree" hypothesis for the species in our sample. Phylomatic is a compilation of previously published phylogenies and its ordinal "backbone" and family resolutions are based on the Angiosperm Phylogeny Website (APweb) [24]. The program matches a species to a reference tree first by "genus", then by "family". Most relationships among and within "genera" are returned as a polytomy due to insufficient resolution within the reference tree at this phylogenetic scale. Branch length information is taken from the single fossil-calibrated molecular divergence time estimates mentioned [25]. We fixed these age estimates and provided dates to undated nodes by distributing

them evenly between nodes with known ages and terminal taxa.

We used R (R Development Core Team, 2009) to obtain slope estimates and R^2 from regression models. Independent contrasts were calculated across our phylogeny using Phylocom (V.4.1; [26]). The method of independent contrasts iteratively calculates trait differences (termed "contrasts") between extant "species" pairs, and subsequently their weighed internal node averages, starting at the tips and moving down to the root of a phylogeny [26]. This calculation transforms the data into $N - 1$ independent data points, each representing an evolutionary divergence. For consistency, the sign of the contrast for the independent variable (e.g., genome size) is set to always be positive with the contrasts of the dependent variable (e.g., pollen width) being compared in the same direction. These contrasts are then standardized by their branch length information to ensure statistically independent data, drawn from a normal distribution with equal variances, which can be analyzed using conventional statistics [27, 28]. Note that since the direction of subtraction in an independent contrast analysis is arbitrary, reversing the direction of subtraction would result in a contrast of the opposite sign. This property gives the expected mean value of zero to all contrasts. Therefore, all regression analyses forced the line through the origin [28].

We calculated a contribution index to examine the proportion of the variation, each divergence contributes to the present-day variation observed in our pollen width data. The contribution index is the product of the amount of variation within a focal clade that is from a particular focal divergence and the amount of the total variation within that focal clade compared with the whole tree (for a detailed discussion, see [29]). That is, large divergences leading to a large number of descendents with a large spread in trait data typically result in higher contribution index scores. Contribution index scores were taken directly from the Phylocom output.

To test whether recent divergences were more likely to lead to dramatic changes in both genome size and pollen width, we preformed two separate but similar analyses. First, we analyzed the independent contrast output for contrasts involving sister tip taxa (i.e., node depth equals 1) and compared this to the complete independent contrast output. The advantage of this approach is that it is completely objective, however, the limitation is that tip taxa contrasts could really be quite divergent because of lack of sister group representation in our dataset. Second, we examined how genome size and pollen width varied genus by genus.

3. Results

Genome size and pollen width information for 464 species was obtained and is summarized in Table 1. The species comprised 50 orders and 85 families of *Spermatophyta* (seed plants; [30]). The angiosperms made up a majority of the dataset (437 out of 464 species) and contained representatives from the major clades: *Magnoliidae* (magnoliids; 2 species), *Monocotyledonae* (monocots; 76 species), and the *Eudicotyledonae* (eudicots; 359 species). Only three families (Cupressaceae, Pineaceae, and Taxodiaceae) represented the extant lineages of gymnosperms (*Acrogymnospermae*; [26]) and all are from the *Coniferae*. The mean 1C DNA estimates for this sample (1C = 22,883.6 Mbp) is comparable to the mean of the acrgymnosperms (1C = 18,111.2 Mbp) taken from the Plant DNA C-values database [19].

Pollen width varied nearly three orders of magnitude, or 2.4-fold, from 7 to 167 μm. The average pollen width was 39.5 μm. *Oenothera biennis* had the largest pollen size (167 μm), while *Myosotis scorpioides* had the smallest pollen size (7 μm) (Table 1). Unlike the 1C DNA data, the mean of the magnoliids was larger (pollen width = 59.0 μm) than the monocots (pollen width = 48.3 μm) and eudicots (pollen width = 35.4 μm). However, the mean of the gymnosperms (pollen width = 67.2 μm) was larger than all three major groups of flowering plants. Of the 21 families that had more than five species represented in our sample, Onagraceae had the largest mean pollen width at 113.9 μm, while Plantaginaceae had the smallest mean pollen width at 23.6 μm.

The combined data sources showed a significant positive trend ($n = 464$, slope = 0.104, $R^2 = 0.096$, P-value<.001, Figure 2(a)). However, our phylogenetically independent contrast analysis suggested that there was a large split between *Angiospermae* versus *Acrogymnospermae* (gymnosperms) for both pollen width and genome size (Table 2), but otherwise, divergences in genome size and pollen width did not co-vary with evolutionary divergences ($n = 197$ contrasts, slope = 0.04, $P > .05$, Figure 2(b)). There were 71 congeneric species pairs in our dataset. Of these, there were significantly more with a positive relationship between genome size and pollen width (44/71, sign test $P < .05$). Twenty-seven of these congeneric pairs had either no relationship (slope = 0) or a negative relationship.

Our literature review of ploidy and pollen width showed consistent reports of pollen width increasing with ploidy (Table 3): results show that pollen size increased by 1.1x to 2x with a doubling of DNA content.

4. Discussion

The consistent strong positive trend that Beaulieu et al. [13] found between plant cell size and genome size is weakly reflected in our analysis of pollen grains. Our regression test was significant across 464 species, but phylogenetically independent species contrasts suggest that the relationship was largely driven by early major divergences during seed plant evolution (between the *Angiospermae* versus *Acrogymnospermae*, e.g., see Table 2 for other significant divergences). At the more microevolutionary level, congeneric species did tend to support the trend of increasing pollen width with increasing genome size, but again, divergences across all taxonomic levels did not support a general evolutionary trend. Previous investigators have found repeated instances of increased pollen width with increasing ploidy levels (Table 3). Our conclusion from these observations is that (1) if there is a relationship between genome size and pollen width, it is more likely exposed at the microevolutionary level, especially when divergences involve variation in ploidy

Table 1: Summary statistics for pollen size and genome size (1C Mbp) for the major groups of plants analyzed in this study.

		Gymnosperms	Angiosperms		
	All Data ($N = 464$)	Coniferae ($N = 27$)	Magnoliidae ($N = 2$)	Monocots ($N = 76$)	Eudicots ($N = 359$)
pollen size					
Smallest	7.00 μm	15.0 μm	44.0 μm	17.0 μm	7.0 μm
Largest	617. 0 μm	108.0 μm	74.0 μm	150.0 μm	167.0 μm
Mean	39.5 μm	67.2 μm	59.0 μm	48.3 μm	35.4 μm
genome size					
Smallest	142 Mb	9727 Mb	784 Mb	294 Mb	142 Mb
Largest	80,262 Mb	31,674 Mb	4753 Mb	80,262 Mb	32,585 Mb
Mean	6540 Mb	22883 Mb	2768 Mb	16,414 Mb	324 Mb

Table 2: Contribution index scores (with rank) for divergences in pollen width and 1C DNA content for the species in our sample.

Rank	Pollen width contribution	Divergences making the largest contribution	1C DNA content rank	1C DNA content contribution
1	0.134	*Angiospermae* versus *Acrogymnospermae*	1	.384
2	0.050	Polytomy at the origin of *Coniferae*	194	<.001
3	0.046	Divergence at the origin of Papilionoideae	99	<.001
4	0.041	Divergence between Lythraceae and Onagraceae	138	<.001
5	0.036	*Magnoliidae* versus *Eudicotyledonae*	3	<.001
6	0.032	Divergence of Fagaceae and the rest of Fagales	81	.002
7	0.031	Polytomy at the origin of eurosid II	127	.002
8	0.030	Divergence between Zingiberales and Poales	175	.002
9	0.026	Divergence between Solanales and Lamiales	35	<.001
10	0.026	Divergence at the origin of Malvaceae	136	.002

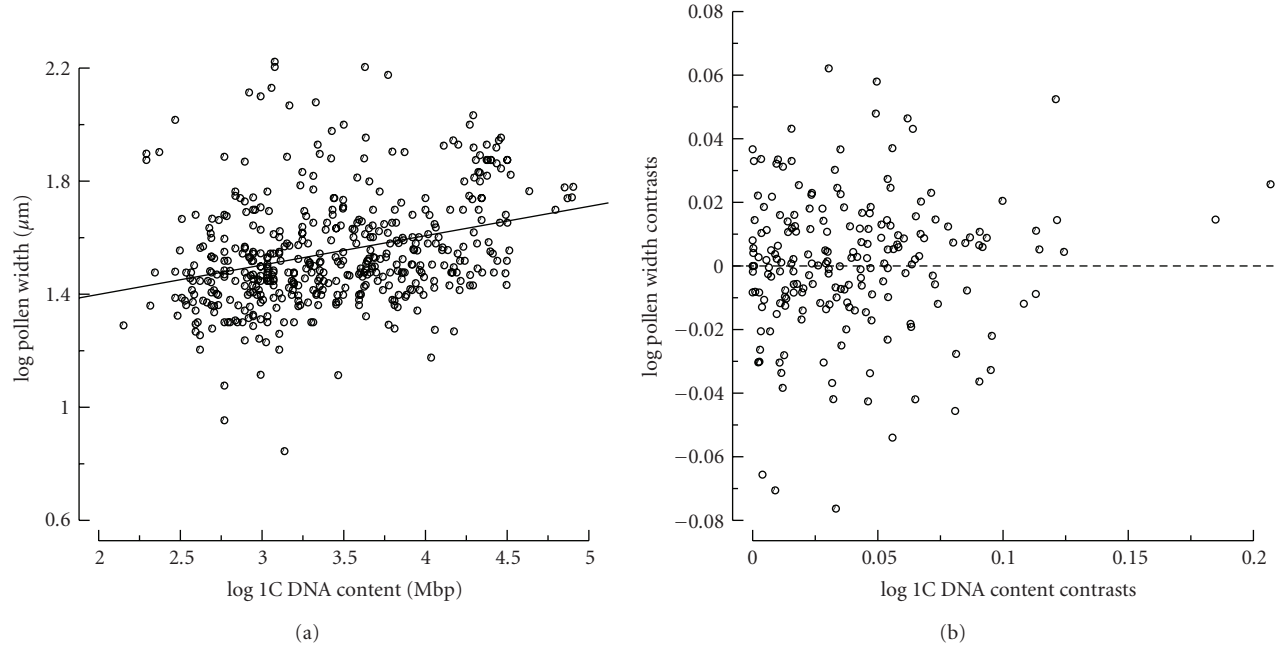

Figure 2: (a) Scatter plot of the significant positive associations between genome size and pollen width. The slope was estimated using conventional least-squares methods that do not incorporate the correlated error structure due to phylogeny. (b) Independent contrast results showing that divergences in 1C DNA content are not associated with divergences in pollen width (open and black points). This result was consistent when isolating the results to just bifurcating sister tip taxa (black points). The unfilled points represent deeper nodes. A line is not shown because the relationship was not significant.

Table 3: Examples of previous studies on the relationship between ploidy and pollen size reported by family, genus, ploidy variation and how doubling DNA content changed pollen volumne. Primary literature sources are also given.

Family	Genus	Species and chromosome numbers	2x the DNA led to:	Source
Boraginaceae	*Lappula*	*deflex*a ($2n = 24$) & *squarrosa* ($2n = 48$)	1.5x to 2x larger pollen	[20]
Convolvulaceae	*Cuscuta*	*epithymum* ($2n = 14$) & *carapestris* ($2n = 56$)	1.2x larger pollen	[22]
Papaveraceae	*Fumaria*	*murialis* ($2n = 30$) & *capreolata* ($2n = 60$)	1.3x larger pollen	[21]
Poaceae	*Andropogon*	various species ($2n = 60$, 120, and 180)	1.2x larger pollen	[14]
Polygonaceae	*Rumex*	*acetosella* ($2n = 14, 28$, and 42)	1.1x to 1.3x larger pollen	[23]
Convolvulaceae	*Ipomoea*	*trifida* with diploid pollen	1.3x larger pollen	[15]
Brassicaceae	*Arabidopsis*	*thaliana* with diploid and tetraploid pollen	1.7x larger pollen	[17]

level, and (2) there was a significant divergence in both genome size and pollen width with the basal divergence between *Angiospermae* versus *Acrogymnospermae*. While the ploidy results suggest a mechanistic link between genome size (of bulk DNA content) and pollen width, the basal divergence between *Angiospermae* versus *Acrogymnospermae* may simply be a coincidence. Our results could also be explained by strong selection for pollen to be small which overwhelms any direct mechanistic link between genome size and pollen size (if there is any).

Natural selection may act strongly on pollen size, especially in relation to pollen dispersal strategies. However, even within species that are primarily bee pollinated, there is considerable variation in pollen size, even though they have very similar genome sizes (e.g., *Luffa* and *Lotus* in Figure 3). In contrast, the sometimes wind-pollinated *Brassica napus* [31] has small pollen (compared to *Luffa* and the rest of our dataset, Figure 3), but *Brassica napus* is also frequently insect pollinated [32]. Complicating matters, in some cases plants are self-compatible and can complete pollination without a vector. *Brassica napus* also fits into this category, it is self-compatible and capable of autonomous pollination [33]. Even in the absence of pollinators, it is able to set half of its seeds in still air and 80% when the stem is shaken [33]. Furthermore, pollination efficiency is considerably affected by local and seasonal environmental conditions [3, 34].

In comparison to other plant phenotypic traits, pollen size varies somewhat less. Pollen size varied in our sample over three orders of magnitude. However, seed mass and genome size vary over ten and five orders of magnitude, respectively [18, 29]. Why is there so little variation in pollen size? There is strong selection favoring small pollen size (as noted above), and likewise, selection pressures against extremely large pollen. Given a size-number trade off in pollen, small pollen may have a higher probability of transport to a receptive stigma both by wind and insect vectors. Perhaps whatever causal factor there is for the relationship between genome size and cell size, it is apparent early after an increase in genome size. But selection pressure favoring small pollen size continually reduces pollen size unless this pressure is relaxed.

The relationship between cell size and genome size may arise from the greater necessity of gene transcripts to service larger cytoplasms [35]. However, pollen is not metabolically active after dehiscence, but rather become so soon after

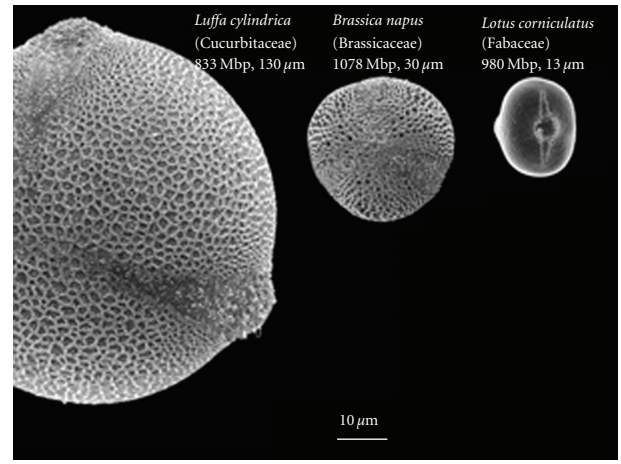

Figure 3: Pollen size can vary considerably (13–130 μm) within a narrow range of genome sizes (833–1078 Mbp).

imbibition and pollen germination. This quiescence makes them quite different from guard cells and other cell types whose sizes have previously been shown to be strongly related to genome size [13, 36]. Perhaps the maximal volume of the pollen tube, after its metabolically active growth stage, may be a better measure of pollen size in this context, not the recently hydrated sphere.

Several measurement errors could have contributed to our weak results. Some of our measurements may have come from unhydrated or incompletely hydrated pollen. In addition, methods of hydration varied from water to glycerine jelly, or silicon oil, each of which can result in different final volumes [37]. Further, various methods of imaging were used, including scanning electron microscopy and light microscopy. There was also no control for the type of pollen reserve (starch or lipids). Each time a new instrument or investigator is involved, there is the possibility that measurements are not standardized/calibrated. Environmental factors can affect pollen size, and not all pollen is exactly spheroidal. However, these are the perils of all meta-analyses. Clearly, more focused and controlled studies are needed to probe the nature of the relationship more fully.

One of the reasons we looked for a relationship between genome size and pollen size was to evaluate the feasibility of using fossil pollen to infer genome sizes over geological time.

Our results suggest that this effort would be difficult and perhaps misleading. Fortunately, the morphology of pollen grains seems to have enough stasis so that species or group level identification is accurate through the paleobotanical record.

Acknowledgments

The authors thank Professor Martina Weber from PalDat (http://www.paldat.org/) for the preparation of Figure 1 and the use of images presented in Figure 3. This project was supported by a sabbatical grant to C. K. and a research stipend for R. C. from the Biological Sciences Department at California Polytechnic State University, San Luis Obispo. They also thank Dr. Gerhard Leubner for hosting them at the University of Freiburg, Germany, where this project was completed.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 390414, 4 pages
doi:10.1155/2010/390414

Research Article

Genome Size Is a Strong Predictor of Root Meristem Growth Rate

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Received 23 December 2009; Accepted 5 March 2010

Academic Editor: Johann Greilhuber

Variation in genome size (GS) has been linked to several facets of the plant phenotype. Recently it was shown that GS is significantly correlated with cell size and the duration of the cell cycle. Here we test the hypothesis that GS might also be a predictor of apical root meristem growth rate (RMGR). We studied eight species of eudicots with varying GS using time-lapse microscopic image analysis. A significant negative exponential relationship was observed between GS and RMGR. Our results show significantly decreased RMGR for large genome species. This relationship represents a significant consequence of GS expansion in plants and may partly explain why genome sizes tend to be small in eudicots. Interestingly, parasitic plants, which do not rely on root growth as much, often have large genomes.

1. Introduction

Genome size (GS) varies by three orders of magnitude in plants [1, 2]. Correlations between GS and several facets of the phenotype and life history of plants have been documented [3, 4]. The strongest relationships are found at the cellular level. For example, GS is positively correlated with cell size [5, 6]. In addition, Francis et al. [7] found a consistent positive relationship between GS and the duration of the cell cycle, which confirmed earlier observations by Bennett [8, 9]. Here we test the hypothesis that GS may be a predictor of meristem growth. We chose to study root meristem because it is relatively easy to model and quantify along a single primary axis. Others have observed correlations between the relative growth rate and genome size [4, 10, 11], but none have looked specifically at growth dynamics (kinematics) in the root meristem.

Apical root meristem growth rate (RMGR) can be modeled with the following equation:

$$\text{RMGR} = N^{(R\,\Delta T)}\left(\frac{\Delta L}{\Delta T}\right), \tag{1}$$

where RMGR is a function of the number of initial cells (N), the division rate of these cells (R), the time it takes for a point to pass through the elongation zone (ΔT), and the change in length of a cell (ΔL) over the change in time (ΔT). All of the independent variables in (1) can be measured using time-lapse microscopic image analysis [12, 13]. We measured root kinematics for seven replicates of eight eudicot species with varying GS to test the hypothesis that GS is a predictor of RMGR (Table 1). Assuming that the number of initial cells remains fairly constant between species, variation in RMGR could be caused by variation in the rates of cell division (R) and/or the elongation rate of these cells ($\Delta L/\Delta T$). Variation in cell division rate would lead to an exponential relationship between genome size and RMGR; variation in cell elongation rate would lead to a linear relationship. Therefore, we tested both exponential and linear models.

2. Methods

2.1. Seed Preparation and Incubation. We measured root growth velocity for eight species (Table 1). All seeds were obtained from Botanical Interests, Inc. (http://www.botanicalinterests.com/). Seeds were sterilized in 6% HCl bleach then rinsed in deionized water. Two seeds of like species were placed on a 100 × 15 mm sterile agar plate. Our agar contained 0.6% (m/v) Phytagel (Sigma-Aldrich, USA) medium containing 0.4% (v/v) nutrient solution (7-9-5 Liquid Grow, Dyna-Gro, San Francisco, CA). The medium was buffered to a pH of 5.8 with 0.05% (m/v) MES. Plates were wrapped with parafilm which

Table 1: Genome size, elongation zone length, and root tip velocity for eight species of eudicots. Data are the means ± SE of at least seven replicates.

Species	Genome size (1C-value - Gbp)	Elongation zone length (μm)	RMGR (μm min^{-1})
Citrullus vulgaris	0.45	2010 ± 316	6.29 ± 1.01
Cucurbita pepo	0.55	2426 ± 354	7.06 ± 0.99
Brassica rapa	0.80	786 ± 41	4.96 ± 0.42
Lycopersicon esculentum	1.01	953 ± 48	5.37 ± 0.41
Cosmos bipinnatus	1.52	108 ± 101	4.60 ± 0.79
Cosmos sulphureus	2.94	937 ± 79	4.02 ± 0.42
Capsicum annuum	3.92	1196 ± 120	2.89 ± 0.26
Capsicum frutescens	5.88	871 ± 63	2.17 ± 0.19

was then perforated to allow gas exchange. Seeds were incubated inside a growth chamber (CMP4030 incubator, Conviron, Winnipeg, Canada) at a 45° angle under 24-hour full light at 25°C. Genome sizes for the species we studied were obtained from the plant DNA C-values database (http://data.kew.org/cvalues/).

2.2. Determining Root Kinematics. We collected microscopic images of roots using a Nikon compound microscope (Nikon Optiphot-2, Nikon, Tokyo, Japan) fitted with a 10/0.30 objective (Nikon Plan, Nikon, Tokyo, Japan) and a digital camera (QICAM-IR, Q-Imaging, Surrey, Canada). Stacks of nine images were taken along the length of the root using a constant interval of 20 to 120 seconds between frames. Each root was assayed between 2 and 14 days after germination. Each plate with a viable root was removed from the incubator and positioned with the root flush to the superior side. Graphite particles were sprinkled along the length of the root using a small paintbrush. These particles were used to produce a background image for each image stack to give a reference for splicing the image stacks together.

We used RootflowRT [13], developed by Tobias Baskin and colleagues, to determine root kinematics from these image stacks (version 2.8, University of Massachusetts-Amherst, available at http://www.bio.umass.edu/biology/baskin/). RootflowRT uses dynamic high contrast image stacks and static background images to create a linear velocity profile of a root. The program determines velocity at various positions by tracking pixels moving along a mapped pathway. The image stacks are first converted to.tif format using IrfanView (http://www.irfanview.com/). The centerline of the fifth image in each stack is then visualized using ImageJ (http://rsbweb.nih.gov/ij/) and points along the line are plotted into the input file. The quiescent center is also entered into the input file and used as the point of origin for velocity determination. The output plot of velocity versus position is a sigmoidal curve representing the three regions of meristematic development. The curve plateaus in the zone of maturation. In this region the root shows little longitudinal growth but begins to grow radially and root hairs and lateral roots appear. For the purposes of this study we focused on the maximum growth rate (the velocity of the quiescent center relative to the zone of maturation).

3. Results and Discussion

There was a significant negative relationship between genome size and RMGR (Figure 1(a)). The trend was remarkably linear across all species. Phylogenetically independent species pairs (*Cosmos* and *Capsicum*) with contrasting genome sizes also showed the same trend: the species with the larger genome size had a significantly slower root growth velocity (Table 1) and a longer elongation zone. The exponential ($F_{df=6} = 97.32$, $P \leq .0001$, $r^2 = 0.94$; Figure 1(a)) and linear ($F_{df=6} = 36.64$, $P \leq .001$, and $r^2 = 0.86$) models were both significant; however, the exponential model was stronger. This result suggests that cell division rate has a larger effect on root meristem growth rate than does variation in cell expansion rate. However, these two models (linear versus exponential) are not mutually exclusive. Decreased cell division rates and elongation rates may both be responsible for decreased RMGR in species with a large GS. If the linear model is correct, the RMGR of eudicots would approach zero at a GS of less than 10 bbp. But many eudicot species are known to have a GS larger than 10 bbp (Figure 1(b)), further supporting the exponential model where changes in cell division rate are the primary driver of changes in RMGR.

Continuous root growth is necessary for plants. Roots must expand into new areas as nutrients are depleted in the nearby soil [14]. As a root grows, it acidifies the environment to facilitate the cotransport of nutrients into the root [15]. This acidification also acts to trigger expansin activity in cell walls, which breaks cross bridges between cellulose microfibrils, leading to cell expansion [16]. Nutrients are absorbed primarily in this area of loosened cell walls in the elongation zone. Therefore, reduction in root growth rate may represent a significant consequence of genome size expansion in plants. A slower RMGR could limit a species to nutrient-rich soils, acidic soils, or require it to find an alternative means of nutrient absorption, such as parasitism [17] or mycorrhizal symbiosis [18]. Currently,

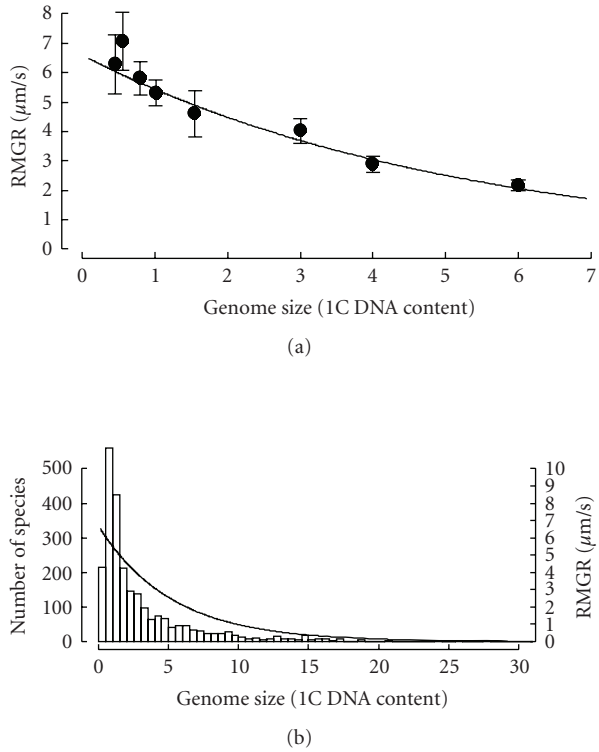

Figure 1: (a) The relationship between genome size (GS) reported in gigabasepairs, and root meristem growth rate (RMGR) for eight eudicot species with the best fit exponential regression line. Error bars are the standard error of the mean for seven replicates for each species. (b) Frequency distribution of genome sizes for eudicots with the best fit line from (a) overlaid.

there are 2,459 species of eudicots that have a known GS. The number of representative species approaches zero at a GS of 25–30 bbp (Figure 1(b)). This is the same GS at which the RMGR approaches zero with our exponential prediction (Figure 1(a)). The only eudicot genus containing species with GS above 30 bbp, *Viscum*, contains parasitic species (mistletoes) which do not use roots to absorb nutrients [17] and can therefore bypass this limitation.

Large genome species may require additional time for DNA synthesis, thus leading to increased cell-cycle duration [5, 6]. However, we hypothesize that it may also involve decreased efficiency of signal transduction for gene expression. For example, a larger GS may increase the time required for a transcription factor to find the proper regulatory element or increase mismatch frequency. This would make gene expression less responsive to endogenous signals. Genes involved in cell-cycle regulation are just one example of an expression system that could be slowed with increasing GS, perhaps resulting in the patterns we observed here. This hypothesis should be tested in a comparative framework. In addition, RMGR should be measured in monocots and gymnosperms, which contain species with significantly larger GS than eudicots.

GS is a nongenic factor that has phenotypic consequences. The patterns we describe here are perhaps the strongest and most significant phenotypic correlations with GS described to date. Further analyses should examine these trends in other plant groups.

Acknowledgment

This project was supported by the Office of Graduate Studies and the Biological Sciences Department at California Polytechnic State University, San Luis Obispo,USA.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 596542, 11 pages
doi:10.1155/2010/596542

Research Article

Heavy Metal Pollution, Selection, and Genome Size: The Species of the Žerjav Study Revisited with Flow Cytometry

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Received 2 March 2010; Accepted 29 April 2010

Academic Editor: Jaroslav Doležel

The Death Valley at Žerjav in northern Slovenia exhibits a gradient of heavy metal pollution in the soil with severe consequences for species richness and composition along this gradient. Recently, a progressive loss of large-genome species in parallel with increasing concentrations of heavy metals has been shown. Here, we have measured the genome size of a near-complete sample of these species with flow cytometry and analysed the correlation of heavy metal pollution with the C- and Cx-values assigned to the test plots. The method of probability analysis was a hypergeometric distribution method. We confirm, on a different methodological basis than previously, that along the pollution gradient, species with high C- and Cx-values are increasingly underrepresented. This lends support to the "large genome constraint hypothesis", predicting that plants with large genomes are at a disadvantage under all aspects of evolution, ecology, and phenotype, because junk DNA imposes a load to the organism.

1. Introduction

The molecular mechanisms that lead to the more than 2000-fold variation in the amount of DNA in higher plants' genomes (Zonneveld [1]), are no longer as mysterious as they were when the term "C-value paradox" was first used (Thomas [2]). The biological significance of this variation, however, is still debated. Several hypotheses are available, of which the Nucleotype Hypothesis has been the most fruitful since its formulation by Bennett [3, 4]. It emphasizes the importance of the physicomechanical properties of the cell nucleus (the nucleotype), in addition to the genotype and the environment, for niche occupation, adaptation, and competitiveness of an organism, in both plants and animals (Gregory [5]). Up to the present, the only well-studied nucleotypic parameters are the C-value and the Cx-value. C-value or holoploid genome size (Greilhuber et al. [6]), that is, the DNA amount contained in the chromosome complement of an organism, is directly and positively correlated with cell size (Bennett [4], Knight and Beaulieu [7]). Cx-value or monoploid genome size, the DNA amount contained in the single basic chromosome set with chromosome number x, has a strong positive influence on cell cycle duration (Bennett [3, 4], Francis et al. [8]). This is clearly a simplification inasmuch as cell and nuclear size vary according to cell type and physiological status (Bennett and Rees [9]). Cell cycle duration is influenced by genetic controls (Francis [10]) and environment, notably temperature (Verma and Lin [11], Verma [12]). Moreover, the distinction between C- and Cx-value is blurred in the case of (palaeo)polyploids with diploidized genomes. Nevertheless, the correlation C-value/cell size/nuclear size holds closely within certain cell types, for example, meristematic and embryonal cells (Greilhuber [13]) and stomata (Knight and Beaulieu [7]), while the correlation Cx-value/cell cycle time has been proven repeatedly (Bennett [3], Verma and Lin [11]). It is noteworthy that (neo)polyploids do not show longer cell cycle duration compared to diploids (Verma and Lin [11]), and sometimes it is even slightly shorter (Bennett [3]). Since the Cx-value of an organism together with the ploidy level determines the C-value, cell cycle duration is also correlated with C-values in global analyses (Francis et al. [8]). Several lines of evidence lend support to the opinion that large genomes are a burden to organisms and restrict

their adaptability. This concept was recently forwarded as the "Large Genome Constraint Hypothesis" (Knight et al. [14]). It would thus be predicted that under stressful environments species with too large a genome increasingly go extinct, either locally or totally. However, the species and genome size spectrum along stress gradients in natural environments has rarely been investigated.

Here, we return to a recent study, in which a number of species and their genome sizes were studied with regard to a gradient of heavy metal pollution along which they grew (Vidic et al. [15]). The locality studied is the "Death Valley", Dolina Smrty, at Žerjav in northern Slovenia. The heavy metal pollution of the soil stems from a former lead smelter and reaches 33.3 g/kg soil at a plot on which plants are still growing. Heavy metals and plant community composition had been analyzed in five test plots (A–E) at distances of 330 m (E), 420 m (D), 520 m (C), 670 m (B), and ~2000 m (A) from the smelter chimney, the last site being the control plot. Diploid herbaceous species had been recorded and collected, and their genome size measured with Feulgen DNA image densitometry. The number of species occurring at the test plots was shown to decrease with increasing pollution, as was the number of species with large genomes (arbitrarily defined as those of the upper quartile of the C-values in the total sample of 70 species). Using a simulation method it was shown that the probability of finding such low frequencies of large genomes by chance decreases with increasing lead pollution. The results were interpreted as being supportive of the Large Genome Constraint Hypothesis (Knight et al. [14]).

The intention behind undertaking a new study on this system is to corroborate the conclusions from the Žerjav study using a technical approach now in much wider use than Feulgen densitometry, that is, flow cytometry using propidium iodide as the DNA stain. Flow cytometry is not only faster, but also more precise thanks to the high number of nuclei measured and other characteristics of the technique (Greilhuber et al. [16]). There was, furthermore, one genome size of *Anemone nemorosa* that was unsupported by literature data [17] and that had to be clarified and possibly corrected. The species measured were collected mostly not in the Death Valley, but this is unproblematic for the present purpose, because genome sizes are fairly stable within a narrow taxon (species at a ploidy level).

To calculate of the chance probability of finding at the test plots the given frequency of large-genome species, we use instead of a lottery simulation method the hypergeometric distribution method. In the Žerjav study by Vidic et al. [15], the probability of finding the observed frequency of large genomes solely by statistical fluctuation was determined by a lottery simulation. But there is a formula to calculate this probability. Distribution of these probabilities is the hypergeometric distribution. Calculation is always advantageous over a lottery simulation, since the result is a priori infinitely precise. A lottery simulation is always an approximation to the calculated value; a repeat will most likely not give the same result. To avoid numerical problems, established statistics software should be used to calculate the hypergeometric distribution.

2. Materials and Methods

2.1. Plant Material. Living plant species as listed in Vidic et al. [15] were collected in Austria, Croatia, and Slovenia (Table 1). Whole plants were wrapped with wet tissue and stored in a plastic bag at the collection sites, and usually kept in the refrigerator for up to one week until investigation with flow cytometry. For *Euphorbia amygdaloides* whole dry fruits were collected. Samples exhibiting potentially problematic compounds (e.g., mucous polysaccharides) were incubated for up to one month for a starving period in the refrigerator, after which time young etiolated leaves or shoots were used for flow cytometry with better success. Herbarium specimens are deposited in WU. Identification of the taxa is based on Fischer et al. [18]. These accessions cover 60 species from 51 genera and 22 families. The C-value of *Orobanche alba* is cited from Weiss-Schneeweiss et al. [19] and those of *Knautia drymeia* and *Scabiosa columbaria* from Temsch and Greilhuber [20] (Table 2). Altogether, 63 species from 54 genera and 24 families are included in this paper.

2.2. Flow Cytometry (FCM). Following the chopping method of Galbraith et al. [21], about 25 mg fresh leaves (dry fruits in case of *Euphorbia amygdaloides*) from each plant sample were cochopped in Otto's buffer I (Otto et al. [22]) together with *Pisum sativum* (1C = 4.42 pg DNA; Greilhuber and Ebert [23]), *Zea mays* (1C = 2.73 pg DNA; Doležel et al. [24]), *Secale cereale* (1C = 7.79 pg DNA; Doležel et al. [24]), or *Solanum pseudocapsicum* (1C = 1.29 pg DNA; Temsch et al. [25]) as the internal standard organisms. The resulting isolated nuclei were filtered through a 30 μm mesh and subsequently incubated with RNase A for 30 minutes at 37°C for digestion of double-stranded RNA. The nuclei were then stained in Otto's buffer II (Otto et al. [22]) containing the fluorochrome propidium iodide (PI, 50 μg/ml) for 1 hour or overnight in the refrigerator. For measurement, a CyFlow ML flow cytometer or a PAII (both Partec, Muenster, Germany) was used. Light sources were for the CyFlow a green laser (532 nm, Cobolt Samba, Cobolt AB, Stockholm, Sweden) and for the PAII a mercury lamp. One preparation was made per individual and from this at least three measurement runs were performed, with 5000 measured particles per run. Usually, the coefficient of variation (CV) was less than 3%, but whenever higher CVs occurred, one or two more runs were added. For each run, the 1C-value was calculated according to the formula: mean fluorescence intensity of the sample organism's G1 nuclei population divided by mean fluorescence intensity of the standard's G1 nuclei population times the 1C-value of the standard organism. The resulting sample values are shown in Table 2.

2.3. Chromosome Counts. Whenever the ploidy levels of our samples had to be clarified, chromosome counts were done on slides made according to protocols for Feulgen densitometric analysis (Greilhuber and Temsch [26]), which was the case in *Campanula rotundifolia, Fragaria vesca, Hieracium murorum, Knautia drymeia, Lathyrus pratensis,*

Table 1: Locality code, country, district, province, village, longitude, latitude, and altitude (m a.s.l.) of each collection site.

Locality Code	District	Province[a]	Village	Longitude[b]	Latitude[b]	Altitude
	Austria					
1	Eisenstadt-U.	B	Siegendorf	16°34′52″	47°46′42″	150
2	Mattersburg	B	Forchtenstein	16°18′30″	47°41′49″	730
3	Neusiedl am See	B	Jois	16°46′10″	47°57′06″	150
4	Oberwart	B	Bernstein	16°17′02″	47°25′20″	610
5	Klagenfurt Land	C	Ferlach	14°15′46″	46°29′09″	640–700[c]
6	Villach Land	C	Villach	13°46′32″	46°34′22″	550
7	Völkermarkt	C	Völkermarkt	14°38′44″	46°41′09″	540
8	Baden	L	Altenmarkt an der Triesting	15°56′01″	48°00′43″	480
9	Baden	L	Furth an der Triesting	16°00′17″	47°58′45″	400–440[c]
10	Baden	L	Pfaffstätten	16°15′22″	48°02′15″	320
11	Bruck/Leitha	L	Götzendorf a. d. Leitha	16°31′10″	48°01′34″	180
12	Bruck/Leitha	L	Hundsheim	16°55′27″	48°07′47″	280–300[c]
13	Krems	L	Krems/Hollenburg	15°41′32″	48°22′15″	320
14	Lilienfeld	L	Kleinzell	15°41′29″	47°58′42″	1050
15	Lilienfeld	L	St. Ägyd am Neuwalde	15°31′04″	47°48′59″	800
16	Melk	L	Dunkelsteinerwald	15°27′05″	48°15′13″	430
17	Melk	L	Texingtal	15°17′18″	48°01′26″	720
18	Mistelbach	L	Mistelbach	16°38′19″	48°33′49″	210
19	Mödling	L	Perchtoldsdorf	16°15′07″	48°07′33″	320
20	Neunkirchen	L	Reichenau a.d. Rax	15°45′40″	47°44′37″	590
21	Neunkirchen	L	Reichenau a.d. Rax	15°46′01″	47°42′51″	1600
22	Neunkirchen	L	Reichenau a.d. Rax	15°46′26″	47°43′10″	1580
23	Neunkirchen	L	Schwarzau im Gebirge	15°38′41″	47°47′38″	790
24	St. Pölten	L	Traismauer	15°45′52″	48°20′48″	190
25	St. Pölten	L	Traismauer	15°45′56″	48°22′12″	190
26	St. Pölten	L	Traismauer	15°47′23″	48°22′00″	185
27	Tulln	L	Sieghardtskirchen	16°03′24″	48°14′07″	240
28	Tulln	L	Sieghardtskirchen	16°04′20″	48°13′47″	280
29	Wiener Neustadt	L	Gutenstein	15°51′00″	47°48′40″	990
30	Wiener Neustadt	L	Miesenbach	15°59′59″	47°51′07″	650
31	Wiener Neustadt	L	Winzendorf-Muthmannsdorf	16°07′21″	47°49′42″	500
32	Wien-Umgebung	L	Purkersdorf	16°11′ 47″	48°11′ 21″	210
33	Bruck an der Mur	S	Bruck an der Mur	15°17′07″	47°23′42″	480
34	Hartberg	S	Waldbach	15°48′12″	47°27′19″	720
35	Liezen	S	Ramsau am Dachstein	13°37′23″	47°27′27″	1800
36	Liezen	S	Weng im Gesäuse	14°32′09″	47°36′53″	850
37	Mürzzuschlag	S	Altenberg an der Rax	15°37′19″	47°41′ 48″	1200
38	Mürzzuschlag	S	Altenberg an der Rax	15°37′ 25″	47°42′16″	1500
39	Mürzzuschlag	S	Altenberg an der Rax	15°38′29″	47°40′53″	1500
40	Weiz	S	Ratten	15°45′54″	47°30′16″	800
41	Lienz	T	Kals am Großglockner	12°37′31″	47°01′35″	1638
42	Gmunden	U	Grünau im Almtal	13°57′00″	47°45′14″	590
43	Vienna	V	Vienna/Lainzer Tiergarten	16°14′40″	48°10′13″	295
44	Vienna	V	Vienna/Gallitzinberg	16°16′24″	48°13′43″	440
45	Vienna	V	Vienna/Lobau	16°28′24″	48°12′19″	155
46	Vienna	V	Vienna/HBV	16°23′02″	48°11′36″	170
	Croatia					
47	Istarska	—	Prascari	13°45′54″	45°16′45″	300

Table 1: Continued.

Locality Code	District	Province[a]	Village	Longitude[b]	Latitude[b]	Altitude
	Slovenia					
48	Koroska	—	Mezica	14°52′10″	46°29′05″	550
49	Notranjska	—	Ilirska Bistrica	14°13′26″	45°34′58″	400
50	Notranjska	—	Postojna	14°00′36″	45°47′34″	780
51	Notranjska	—	Postojna	14°12′03″	45°47′49″	580
52	Primorska	—	Divaca	13°58′37″	45°39′43″	450
53	Stajerska	—	Ptuj	16°00′00″	46°22′12″	200

[a]Abbreviations of Austrian provinces. B = Burgenland, C = Carinthia, L = Lower Austria, S = Styria, T = Tyrol, U = Upper Austria, V = Vienna, [b]World Geodetic System 1984, [c]Collection areas' positions are given as the coordinates of the mid point.

Table 2: Chromosome numbers, ploidy levels, and mean C-values (Gbp and pg) from Vidic et al. [15] compared to the mean C- and Cx-values of the present study, and the locality code. Species are listed in alphabetical order.

Vidic et al. [15]					Present study						
					Taxon mean values			Accession data			
Taxon	2*n*	Ploidy	2C (Gbp)	1C (pg)	1C (pg)	S.D. (pg)	1Cx (pg)	Locality Code	1C (pg)	S.D. (pg)	*N*[a]
Anemone nemorosa L.	30	4*x*	10.80	5.52	19.479	0.359	9.740	48	19.073	0.267	5
								16	19.610	0.692	5
								46	19.755	0.058	5
Arabis hirsuta (L.) Scop.	32	4*x*	1.34	0.69	0.454		0.227	13	0.454	0.002	5
Aremonia agrimonoides (L.) DC.	35	5*x*	2.43	1.24							
Aruncus dioicus (Walter) Fernald	18	2*x*	0.42	0.21	0.174	0.002	0.174	27	0.173	0.001	3
								53	0.176	0.001	3
Asarum europaeum L.	26	2*x*	12.37	6.32	6.646	0.138	6.646	46	6.554	0.019	3
								53	6.579	0.016	3
								9	6.805	0.032	3
Aster bellidiastrum (L.) Scop.[b]	18	2*x*	3.12	1.60	1.517		1.517	5	1.517	0.008	3
Astragalus glycyphyllos L.	16	2*x*	1.63	0.83	0.751		0.751	26	0.751	0.002	3
Betonica alopecuros L.	16	2*x*	4.43	2.26	2.421		2.421	37	2.421	0.051	3
Biscutella laevigata L.[c]	36	4*x*	3.62	1.85	1.828		0.914	35	1.828	0.004	3
Buphthalmum salicifolium L.	20	2*x*	4.72	2.41	2.490		2.490	19	2.490	0.001	3
Campanula cespitosa Scop.	34	2*x*	2.64	1.35	1.093	0.009	1.093	6	1.083	0.006	3
								36	1.086	0.005	3
								22	1.094	0.003	3
								20	1.100	0.001	3
								20	1.103	0.001	3
Campanula rotundifolia L.	34	2*x*	2.62	1.34	1.096		1.096	7	1.096	0.005	3
Campanula thyrsoides L.	34	2*x*	5.25	2.68	2.365		2.365	37	2.365	0.005	3
Carlina acaulis L.	20	2*x*	10.27	5.25	5.094		5.094	14	5.094	0.023	3
Centaurea fritschii Hayek	20	2*x*	3.53	1.80	1.681[d]		1.681	19	1.681	0.015	3
Cirsium erisithales (Jacq.) Scop.	34	2*x*	2.38	1.22	1.056		1.056	30	1.056	0.011	3
Cruciata glabra (L.) Ehrend.	22	2*x*	1.45	0.74	0.681		0.681	2	0.681	0.003	3
Cyclamen purpurascens Mill.	34	2*x*	6.03	3.08	3.303	0.002	3.303	9	3.302	0.004	3
								53	3.305	0.010	3
Digitalis grandiflora Mill.	56	2*x*	2.55	1.30	1.150		1.150	26	1.150	0.005	5
Erysimum sylvestre Scop.	14	2*x*	0.55	0.28							
Eupatorium cannabinum L.	20	2*x*	4.83	2.47	2.577	0.033	2.577	50	2.554	0.004	3
								11	2.601	0.005	3

Table 2: Continued.

Vidic et al. [15]					Present study						
					Taxon mean values			Accession data			
Taxon	2*n*	Ploidy	2C (Gbp)	1C (pg)	1C (pg)	S.D. (pg)	1Cx (pg)	Locality Code	1C (pg)	S.D. (pg)	*N*[a]
Euphorbia amygdaloides L.	20	2*x*	5.38	2.75	3.508		3.508	36	3.508	0.006	3
Euphorbia cyparissias L.	40	4*x*	2.17	1.11							
Fragaria vesca L.	14	2*x*	0.31	0.16	0.246	0.006	0.246	9	0.243	0.002	3
								27	0.254		1
Galium anisophyllon agg. *austriacum*	22	2*x*	1.00	0.51							
Galium mollugo L.[e]	44	4*x*	3.69	1.89	1.885		0.942	49	1.885	0.013	3
Galium verum L.	44	4*x*	3.80	1.94	1.887	0.047	0.943	4	1.775	0.001	3
								3	1.851	0.002	3
								24	1.869	0.004	3
								19	1.889	0.004	3
								11	1.894	0.006	3
								18	1.894	0.006	3
								32	1.914	0.004	3
								1	1.922	0.007	3
								49	1.929	0.004	3
								47	1.932	0.012	3
Galium x pomeranicum	44	4*x*	3.66	1.87	1.888	0.007	0.944	3	1.882	0.002	3
								32	1.885	0.010	3
								18	1.895	0.009	3
Gentiana asclepiadea L.	44	4*x*	6.45	3.30	3.140	0.023	1.570	53	3.124	0.003	3
								39	3.156	0.008	3
Gentianella ciliata (L.) Borkh.	44	4*x*	20.07	10.26	9.004		4.502	42	9.004	0.014	3
Helleborus niger L.	32	4*x*	29.87	15.27	14.728	0.185	7.364	5	14.552	0.054	3
								23	14.683	0.087	3
								9	14.686	0.168	3
								15	14.990	0.045	3
Hepatica nobilis Schreber	14	2*x*	34.45	17.61	16.709		16.709	12	16.709	0.157	3
Hieracium murorum L.	27	3*x*	9.02	4.61	5.706		3.804	37	5.706	0.045	3
Hippocrepis comosa L.	28	4*x*	3.73	1.91							
Hypericum perforatum L.	32	4*x*	1.59	0.81	0.783	0.065	0.392	19	0.737	0.004	3
								46	0.829	0.002	3
Knautia drymeia Heuff.	40	4*x*	6.38	3.26	7.031[f]		3.516				
Laserpitium peucedanoides L.	22	2*x*	3.03	1.55							
Lathyrus pratensis L.	14[g]	2*x*	8.89	4.54	5.951		2.975	44	5.951	0.010	3
Leontodon hispidus L.	14	2*x*	4.46	2.28	2.188	0.057	2.188	33	2.123	0.026	3
								33	2.212	0.002	3
								13	2.230	0.024	3
Leontodon incanus (L.) Schrank	8	2*x*	2.39	1.22	0.956		0.956	36	0.956	0.001	3
Lotus corniculatus L.	24	4*x*	2.48	1.27	1.235		0.618	13	1.235	0.003	3
Medicago lupulina L.	16	2*x*	1.35	0.69	0.649		0.649	13	0.649	0.003	3
Minuartia gerardii (Willd.)	24	2*x*	1.98	1.01	1.092		1.092	21	1.092	0.004	3
Origanum vulgare L.	30	2*x*	1.79	0.92	0.718		0.718	12	0.718	0.004	3
Orobanche alba Steph. ex Willd.	38	2*x*	6.03	3.08	2.979[h]		2.979				
Petrorhagia saxifraga (L.) Link	60[i]	4*x*	2.20	1.12	0.648		0.648	3	0.648	0.001	3
Phyteuma orbiculare L.	22	2*x*	2.26	1.16	1.136	0.024	1.136	5	1.119	0.001	3
								29	1.153	0.007	3

Table 2: Continued.

Vidic et al. [15]					Present study						
					Taxon mean values			Accession data			
Taxon	2*n*	Ploidy	2C (Gbp)	1C (pg)	1C (pg)	S.D. (pg)	1Cx (pg)	Locality Code	1C (pg)	S.D. (pg)	*N*[a]
Pimpinella saxifraga L.	20	2*x*	8.33	4.26	3.823		3.823	28	3.823	0.015	3
Plantago major L.	12	2*x*	1.39	0.71	0.712	0.002	0.712	53	0.710	0.000	3
								11	0.713	0.003	3
Plantago media L.	24	4*x*	5.11	2.61	2.782	0.084	1.391	12	2.727	0.006	3
								31	2.741	0.006	3
								50	2.878	0.006	3
Polygala amara ssp. *brachyptera*	34	2*x*	1.05	0.54	0.422		0.422	4	0.422	0.002	3
Potentilla erecta (L.) Räuschel	28	4*x*	0.89	0.46							
Primula vulgaris Huds.	22	2*x*	1.14	0.58	0.470	0.006	0.470	8	0.463	0.001	3
								53	0.471	0.002	3
								46	0.475	0.009	3
Prunella vulgaris L.	28	2*x*	1.27	0.65	0.650	0.016	0.650	51	0.639	0.003	5
								11	0.661	0.002	3
Ranunculus nemorosus DC.	16	2*x*	9.86	5.04	6.147		6.147	41	6.147	0.014	3
Salvia glutinosa L.	16	2*x*	2.31	1.18	1.071	0.023	1.071	53	1.055	0.001	3
								25	1.087	0.006	5
Scabiosa columbaria L.	16	2*x*	2.12	1.08	1.072[j]		1.072				
Silene alpestris Jacq.	24	2*x*	2.64	1.35	2.210	0.003	2.210	37	2.208	0.004	5
								36	2.212	0.009	3
Silene dioica (L.) Clairv.	24	2*x*	5.40	2.76	2.734	0.003	2.734	34	2.732	0.012	3
								40	2.736	0.007	3
Silene nutans L.	24	2*x*	4.96	2.54	2.385	0.005	2.385	17	2.381	0.004	3
								12	2.388	0.003	3
Silene pusilla Waldst. & Kit. s. lat.	24	2*x*	2.82	1.44	1.324		1.324	37	1.324	0.002	3
Solidago virgaurea L.	18	2*x*	1.77	0.90	1.134		1.134	38	1.134	0.005	3
Symphytum tuberosum L.	64	8x	5.73	2.93	2.745		0.686	43	2.745	0.014	3
Taraxacum officinale Weber in Wiggers	16	2*x*	2.50	1.28	1.254		1.254	45	1.254	0.004	3
Thlaspi praecox Wulf.	14	2*x*	0.66	0.34	0.262	0.024	0.262	46	0.245	0.001	3
								48	0.279	0.002	3
Tussilago farfara L.	60	2*x*	3.78	1.93	1.736	0.048	1.736	12	1.702	0.005	3
								12	1.770	0.004	3
Verbascum austriacum Schott ex Roem & Schult.[k]	26	2*x*	0.87	0.44	0.357		0.357	3	0.357	0.003	5
Veronica officinalis L.	36	4*x*	2.31	1.18	0.901		0.451	30	0.901	0.006	3
Vincetoxicum hirundinaria Med.	22	2*x*	0.71	0.36	0.311	0.006	0.311	12	0.307	0.003	3
								52	0.316	0.001	3
Viola hirta L.	20[g]	2*x*	1.70	0.87	1.511		0.755	10	1.511	0.069	3

[a]*N* = number of runs
[b]*Aster bellidiastrum* = *Bellidiastrum michelii* Cass.
[c]Collected by C. König
[d]*Centaurea fritschii* substituted by *Centaurea scabiosa* L. ssp. *scabiosa*
[e]Tetraploid *Galium mollugo* is now *G. album* Mill.
[f]*Knautia drymeia*: The ploidy level of Vidic et al. [15] should be reinvestigated. The present value is from Temsch and Greilhuber [20]
[g]The present samples of *Lathyrus pratensis* (2*n* = 28) and *Viola hirta* (2*n* = 40) are tetraploid
[h]Weiss-Schneeweiss et al. [19]
[i]The present sample of *Petrorhagia saxifraga* is diploid (2*n* = 30)
[j]The present value is from Temsch and Greilhuber [20]
[k]*Verbascum austriacum* = *Verbascum chaixii* Vill. subsp. *austriacum* (Roem. & Schult.) Hayek

Origanum vulgare, Petrorhagia saxifraga, Plantago major, Plantago media, Veronica officinalis, and *Viola hirta.* Proliferating parts of these species were fixed in 3 : 1 methanol/acetic acid (3 : 1) overnight at 7°C, or in formaldehyde (FA, 4% in Sörensen buffer) for two hours followed by 3 : 1 fixation, and transferred to 96% ethanol for further storage. Feulgen stained mitotic configurations were analyzed for chromosome number (Table 2).

2.4. Data Analysis. Vidic et al. [15] described a decrease in species having large nuclear characters with decreasing distance of the sample plots to a source of heavy metal pollution. Large nuclear characters are the upper quartile of C-values, Cx-values, chromosome number, and mean chromosomal DNA content. In Vidic et al. [15] a randomization test was used to determine the probability for large nuclear characters equal or less than observed for each plot. The probability can, however, be calculated directly: The number of distinct samples of size K out of a lot of size N is given by the binomial coefficient $Q = \binom{N}{K} = N!/K!(N-K)!$. (A number lottery is a common example: There are $\binom{45}{6}$ distinct patterns of 6 numbers out of 45). If we only consider large nuclear characters, there are $\binom{L}{i}$ distinct patterns of size i for L large characters in the entire lot. The number of samples of size K with exactly i large characters is given by $Q_i = \binom{L}{i}\binom{N-L}{K-i}$, since each of the large character patterns can be combined with all possibilities to select K-i small characters out of N-L in the lot. The probability of finding exactly i large genomes in a sample of size K is $p_i = Q_i/Q$. This is the hypergeometric probability distribution. The probability of finding i or less large genomes (cumulative probability) as described in Vidic et al. [15] is $\sum_{k=0}^{L} p_k$. Obviously $\sum_{k=0}^{L} p_k = 1$, which simply means, the number of large genomes in the sample is less or equal to L, the number of large genomes in the lot.

Binomial coefficients are composed of the factorials of the occurring numbers. These can be very large numbers, which are likely to exceed the range of representable numbers in a computer system, or at least substantial loss of precision has to be expected. Therefore it is strongly recommended to use established statistics software to calculate such probability distributions. Here we used SAS 9.1 (SAS [27]). As an alternative to commercial software we made trial runs based on prime factor decomposition of the factorials to circumvent the problems with large numbers. We got the same results as in SAS. The accordance of our data with Vidic et al. [15] was visualized by a Bland-Altmann-plot (Figure 1).

3. Results

3.1. Data. Table 2 contains data from 63 species, of which 60 are original and have been measured with flow cytometry and 3 further were taken from published sources (Temsch and Greilhuber [20], Weiss-Schneeweiss et al. [19]). These are 90% of the species investigated by Vidic et al. [15]. Chromosomes were counted in nine species in which more than one ploidy level occurs and a deviation between our data and those of Vidic et al. [15] was recognized. In three species the ploidy level given in Vidic et al. [15] and here in Table 2 is not the same. Different ploidy levels (recognized by chromosome counts) between the two studies were found in the following taxa: *Lathyrus pratensis*, *Petrorhagia saxifraga*, and *Viola hirta* (Table 2). In this case, we corrected our C-values for ploidy level given for the Žerjav plants in the further calculations, assuming that the actual C-value of these plants closely approaches this estimate.

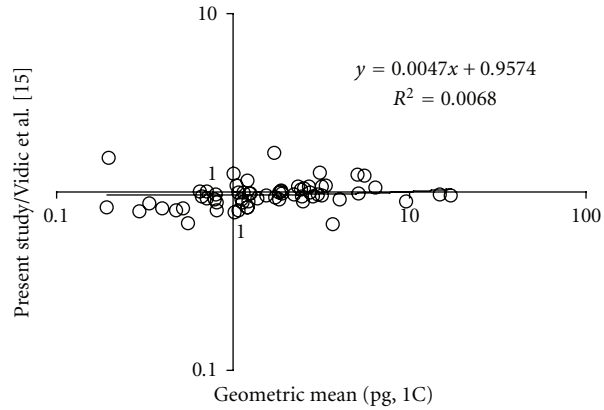

Figure 1: Modified Bland-Altmann plot of the present data versus those of Vidic et al. [15] without the value of *Anemone nemorosa* and with incongruent ploidy levels corrected. The values are displayed on logarithmic axes.

A comparison of the data of Vidic et al. [15] and those of the present study shows, besides a general agreement in many points, some striking deviations from the expectation. There is in the first instance *Anemone nemorosa*, of which the low 1C-value of 5.5 pg given in Vidic et al. [15] could not be confirmed with material from Žerjav and can only be a mismeasurement, because it is well below the 1C-value of diploids, which are expected to have about 10 pg (Zonneveld in Bennett and Leitch [17]). We also measured material from the Botanical Garden in Vienna (HBV) and from one accession in Lower Austria with congruent results (19.48 pg), very similar to the C-value of Zonneveld et al. [28] (1C = 19.05 pg). *Anemone nemorosa* was therefore excluded from some statistical analyses. Another case of deviation is *Knautia drymeia*. Vidic et al. [15] give 1C = 3.26 pg at $2n = 4x = 40$ (which is possibly an error and should read $2n = 20$), while we measure 1C = 7.031 pg at $2n = 4x = 40$ (Temsch and Greilhuber [20]).

The variation within the 1C-values ranged 114.6-fold from 0.17 pg in *Aruncus dioicus* to 19.48 pg in the tetraploid *Anemone nemorosa*. 1Cx-values varied 98.3-fold from 0.17 pg in *Aruncus dioicus* to 16.71 pg in *Hepatica nobilis*.

3.2. Comparison of the Data Sets. Among the 62 species for which agreement was established in regard to ploidy level, 38% agree within +/−0 to 5%, 24% within +/−5 to 10%, 19% within +/−10 to 20%, and 19% are more strongly deviating. The average ratio (present study/Vidic et al. [15]) over these species is 0.97. 14 C-values in Vidic et al. [15] are lower than the present ones. The correlation (C-values

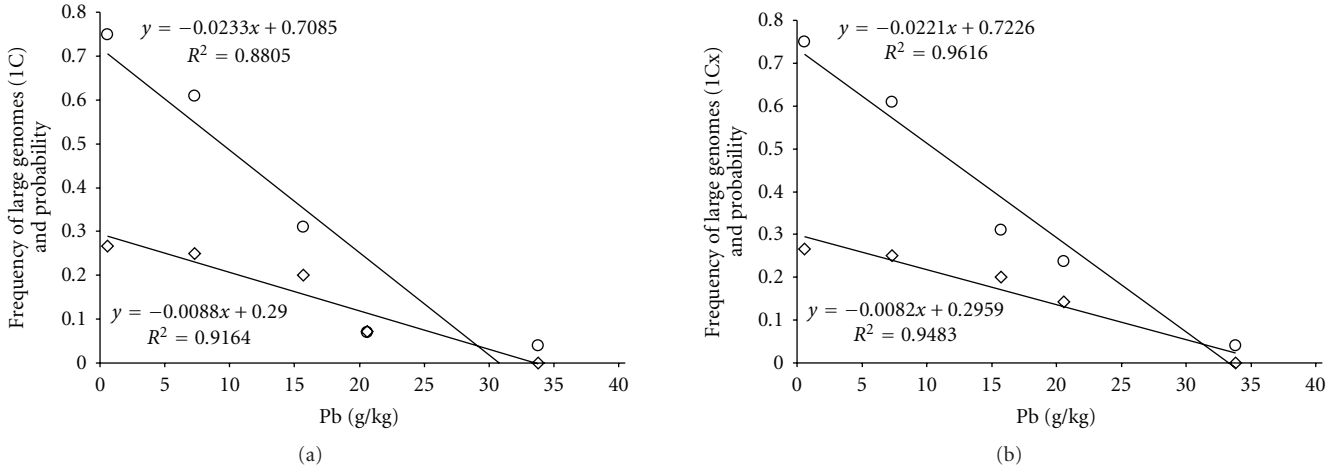

Figure 2: Correlation of lead concentration at the five test plots (from left to right A–E [15]) with (i) percent large genomes (diamonds) and (ii) probability of finding by chance that number or fewer large genomes (circles).

corrected for ploidy and without *A. nemorosa*) yielded a correlation coefficient $r = 0.9917$.

3.3. Probability Analysis. Vidic et al. [15] addressed the question of whether the degree of heavy metal pollution along a gradient has an influence on the C-value and the Cx-value spectra of the species living on five test plots. Since the number of species decreases with increasing pollution, the crucial point is whether the observed shift of the C-value composition towards lower C-values is stronger than expected under the condition of randomness. Vidic et al. [15] found a significant decrease of species with large genomes along increasing pollution using a lottery simulation technique. The present approach relies on the direct calculation of the probabilities of the occurrence of large nuclear characters.

3.3.1. Our Data Set Analysed Using Hypergeometric Distribution. There are minor differences between the values plotted in Vidic et al. [15, Figure 2(c)] and the results that we recalculated applying our method to the data of Vidic et al. [15]. We calculated the probability from the composition of the entire set (hypergeometric distribution), wheras Vidic et al. [15] counted the probability from randomly drawn samples. Their method is an estimate, strictly speaking, which would explain the deviation. It is unlikely that Vidic et al. [15] would obtain exactly the same results in case of a repeat of their method. In our calculation the probabilities for having found a random genome size spectrum decrease with increasing lead concentration at the plots. At the highest lead concentration, the probability level of 4.1% is reached with the C- and the Cx-value spectrum.

Using the limits for large genomes (upper quartile) from Vidic et al. [15] the result appears slightly more significant. This probability should not be misunderstood as significance of a statistical test. Rather it is a characterization of the detrimental effect of pollution on large genomes: a low probability makes it unlikely that pollution has no effect on the phytocoenosis at this plot. The species and data set of Vidic et al. [15] analysed with hypergeometric distribution result in similar Figures (Figures 2(a) and 2(b)) as in [15, Figure 2], reaching 11.3% probability for the species composition at plot E [15] for 1Cx-values and 1.3% for 1C-values.

Regarding the frequency of large genomes, there are slight differences between the limits set by us and by Vidic et al. [15] because large genomes are defined as the upper quartile of the genome size distribution. In our data set, large genomes are those with 1C > 2.781 pg and 1Cx > 2.576 pg. The progressive elimination of large genomes (C- and Cx-values) with increasing heavy metal pollution and decreasing distance to the lead smelter is clear with both data sets (Figures 3(a)–3(c), Table 2), but much more convincing with the present 1Cx data. The deficit of large 1Cx-values at plot A in Vidic et al. [15] data seems to result from wrong ploidy levels in some species. The frequency of polyploid genomes also decreases but with less regularity (Figures 4(a) and 4(b)).

The correlations between heavy metal pollution at test plots on the one hand, and % species with large genomes ($P = .005$, $r = -0.974$) and the probabilities of hypergeometric distribution ($P = .003$, $r = -0.981$), on the other hand, are negative and statistically significant.

4. Discussion

Vidic et al. [15] demonstrated a strong negative correlation between heavy metal content of the soil and the proportion of plant species with large genomes along a gradient of pollution caused by the emissions of a lead smelter. The conclusion was that genome size is associated with differential survival of species in this extreme environment and that species with large genomes are at a disadvantage.

The present paper is in some respect a control investigation for the Žerjav study by Vidic et al. [15] using a different methodology for genome size measurement, that is, more precise flow cytometry instead of the error-susceptible

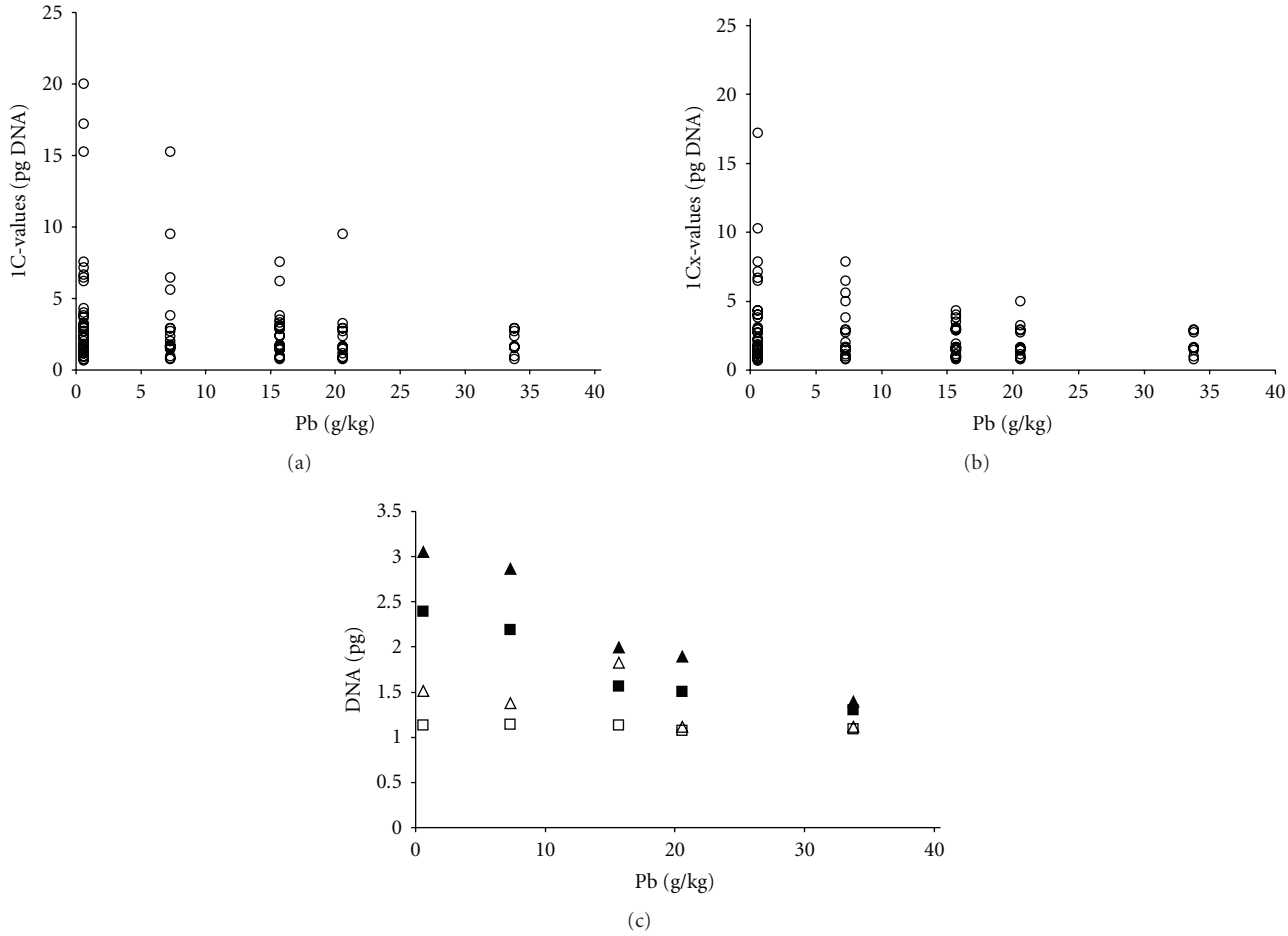

Figure 3: (a) Distribution of the 1C-values and (b) 1Cx-values of the taxa as they occur at the sample plots, and (c) the median 1Cx-values (empty squares), the mean 1Cx-values (filled squares), the median 1C-values (empty triangles), and the mean 1C-values (filled triangles).

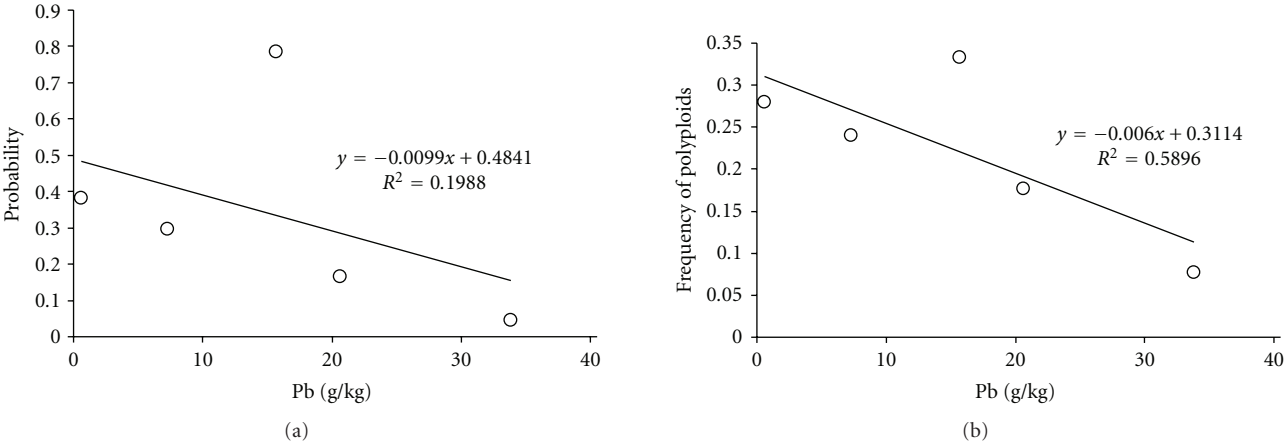

Figure 4: (a) Probability of finding by chance that number or fewer polyploids, and (b) percent polyploids at the five test plots.

Feulgen densitometry, and for calculation of probabilities for large genome frequencies, that is, a hypergeometric distribution method instead of a simulation method. Our material originated mostly not from Žerjav. However, the results are representative for the Žerjav locality because genome sizes are generally very stable within a species.

Our results confirm the findings of Vidic et al. [15] and exhibit in some regard even clearer trends. The species characterized by large genomes (i.e., upper quartile of the genome sizes) out of the complete species spectrum are increasingly removed in parallel with heavy metal pollution until none of these species is left in the most polluted site.

This clearer trend, compared to Vidic et al. [15], may be the consequence of removing a few possible errors from the Žerjav data set (mainly correction of a mismeasurement in *Anemone nemorosa* and possible misidentification of ploidy level in *Knautia drymeia*), and a general improvement of the genome size data using a more precise measurement method. Notably, *Anemone nemorosa* ranks highest in genome size and occurs only at the control site (plot A), while it is only fifth-highest in the data set of Vidic et al. [15].

Vidic et al. [15] considered only C-values, while we also consider Cx-values. With these, we find the same trends as with C-values inasmuch as the frequency of large monoploid genomes and the hypergeometrically distributed probability decrease continuously from plot A to plot E. The frequency of polyploid genomes also decreases finally, but plot C is the highest and violates any significance. The observed trends, therefore, cannot be attributed either to the C-value or to the Cx-value alone.

The positive correlation between radiosensitivity and genome size in plants described by Sparrow and Miksche [29] seems to be most relevant for the present results, because direct genotoxic effects are involved in both studies. A high frequency of chromosome aberrations has been observed by Druškovič [30] in plants from the Death Valley, Dolina Smrti at Žerjav. It seems plausible to us that DNA damage with its various consequences for cell division and cell function is the primary cause for the exclusion of large genomes at the more heavily polluted plots. Once a nucleus is hit, the damage is realized irrespective whether the nucleus is big or small, but large nuclei and especially mitotically and meiotically active ones are a larger target for damage and receive more hits.

Apart from chemical stress, also natural stress such as extreme temperatures and low precipitation seem to favour plants with small genomes rather than large ones in certain floras, whereas the species with largest genomes occur preferably in mesic areas (Knight and Ackerly [31]). This and other evidence led Knight et al. [14] to propose the Large Genome Constraint Hypothesis, which predicts that "plants with large genomes are at a disadvantage under all aspects of evolution, ecology and phenotype because large genomes are inflated with unnecessary junk DNA whose replication and maintainance imposes a load to the organism" (Knight et al. [14]).

In line with this hypothesis is the observation of Vinogradov [32] that red-list species more often have large genomes than non-endangered species. Gruner et al. [33] report slower root growth in species with large genomes, which may be one factor of risk for endangered plant species. The analyses by Bennett et al. [34] in weeds and nonweeds showed that weeds and especially the most aggressive weeds have smaller genomes than nonweeds, which indicates that competitive stress brings plants with larger genomes into a disadvantage.

Šmarda et al. [35] investigated differential seedling survival in a tetraploid *Festuca pallens* population with up to 1.189-fold genome size variation at constant chromosome number of $2n = 28$. In this case, during development of the plants those with the lowest and the highest C-values were eliminated under conditions of intraspecific competition for resources. This case of stabilizing selection could be explained by a disadvantage for karyotypes with too many deletions or duplications and is of a different character than the selection phenomena observed under chemical or ecological stress.

Acknowledgments

The authors firstly thank Marina Dermastia and Tatjana Vidic for kindly supporting this investigation with plant material (*Anemone nemorosa*) and for valuable discussions and Jasna Dolenc Koce (Ljubljana) and Markus Koch (Heidelberg) for seeds of *Thlaspi praecox*. The authors also thank Friedrich Ehrendorfer, Irmgard Greilhuber, Josef Greimler, Walter Gutermann, Elvira Hörandl, Christiane König, Harald Niklfeld, and Hermann Voglmayr for providing material and for helping with the identification, and Martina Mittlböck and Harald Heinzel for the valuable discussion on data analysis.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 797246, 19 pages
doi:10.1155/2010/797246

Research Article

Genome Size Study in the Valerianaceae: First Results and New Hypotheses

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Received 28 January 2010; Accepted 10 May 2010

Academic Editor: Jan Suda

The purpose of this study is to provide a new focus to contribute, from the perspective of genomic evolution, towards a better understanding of the Valerianaceae evolutionary history. Chromosome numbers were determined by Feulgen staining in 24 populations of 18 species (first count for *Valerianella multidentata*, $2n = 2x = 14$–16), and DNA contents were assessed by flow cytometry in 74 populations of 35 species (first assessments in all taxa but *Centranthus ruber*). A molecular phylogeny based on the *trn*L-*trn*F and including 41 new sequences was established, with the first DNA sequence for *Centranthus nevadensis*, *Valeriana rotundifolia*, *V. saxatilis*, *Valerianella multidentata*, and *V. turgida*. This work is the first large genome size study devoted to the Valerianaceae, showing a range of DNA amounts from 2C = 0.39 pg (*Valerianella turgida*) to 2C = 8.32 pg (*Valeriana officinalis*). At the family level, changes in basic chromosome number and genome size coincide with or precede major shifts in the evolutionary history of the group, such as those concerning stamen number and floral symmetry.

1. Introduction

The family Valerianaceae (currently considered within the Caprifoliaceae s.l.; [1]) comprises ca. 400 species of which approximately 200 are included in *Valeriana* L., the biggest genus of the order Dipsacales. The Valerianaceae are cosmopolitan in their natural distribution, with the exceptions of Australia and the Pacific islands, where they were introduced, and can be nowadays considered as naturalized. Several of their representatives have economic interest as medicinal (e.g., *V. officinalis* L.), edible (e.g., *Valerianella* Mill., corn salad or lamb's lettuce), or ornamental plants (e.g., *Centranthus* DC.). The family always roused important scientific interest, and its study has broadly benefited from the new molecular techniques, especially the phylogenetic reconstructions. Recent phylogenies based on DNA sequencing [2–7] have considerably modified the traditional classification of the family [8–12]. Molecular results recognize only six genera: *Centranthus*, *Fedia* Gaertn., *Nardostachys* DC., *Patrinia* Juss., *Valeriana* (including *Aretiastrum* DC., *Astrephia* Dufr., *Belonanthus* Graebn., *Phyllactis* Pers., *Porteria* Hook., and *Stangea* Graebn.), and *Valerianella*. *Plectritis* (Lindl.) DC. is nested amongst South American *Valeriana* [5, 6]. Moreover, Bell [7] suggested a possible further taxonomic treatment of the family considering *Fedia* as a synonym of *Valerianella*. New genera may also be described for some of the *Valeriana* species that do not group with their congenerics in the phylogenetic reconstructions and make the genus *Valeriana* paraphyletic in its current circumscription. These are *Valeriana longiflora* Willk., which appears closely related

to the genus *Centranthus*, and also *Valeriana celtica* L. and *V. hardwickii* Wall., branched somewhere between *Nardostachys* and *Valerianella* plus *Fedia* clades [4, 5].

Morphology in Valerianaceae is of great interest for its impressive diversity of forms, mainly resulting in adaptations to a wide range of ecological conditions (from the sea board to Andean páramos at 4000 m), and concerning both vegetative and reproductive parts. This diversity has been especially well studied from the inflorescential structure perspective, with a common basic type, the thyrse, and very different forms and levels of complexity [13, 14]. Family Valerianaceae stands out in Dipsacales by presenting four different stamen numbers in a series (mainly four stamens in *Patrinia* and *Nardostachys*, three in *Plectritis*, *Valerianella* and *Valeriana*, two in *Fedia*, and one in *Centranthus*), while the other families show one or two distinct stamen numbers [15, 16]. These features, along with some cases of strong corolla zygomorphy (in *Centranthus*, with 4 : 1 petals orientation; in *Fedia*, with 2 : 3 petals orientation) make the family very attractive for studying the genetic base of floral characters, which has been initiated through an evolutionary-developmental approach by [16, 17]. Evo-devo studies highlight the crucial importance of duplication events in the evolution of genes involved in developmental processes (e.g., [18, 19]). These events often correspond to whole duplication genome ([20], and references therein), making the karyological and cytogenetic data essential for understanding many evolutionary processes, as, for example, the floral morphological changes.

Nevertheless, karyological and cytogenetic data in Valerianaceae are basically limited to chromosome counts. The Valerianaceae exhibit a dysploid series of five basic chromosome numbers [21]: $x = 15$ in American *Valerianella*, $x = 13$ in *Nardostachys*, $x = 11$ in *Patrinia* and *Valeriana celtica*, $x = 8$ in *Centranthus*, *Fedia*, *Valeriana* and *Valerianella*, and $x = 7$ in *Valeriana* and *Valerianella*. Polyploidization events are common, some genera being exclusively polyploid, such as *Centranthus* (tetraploid) or *Fedia* (tetraploid and hexaploid), while species of *Patrinia* and *Valeriana* can exhibit various ploidy levels, from diploid to octoploid. Hence, both polyploidy and dysploidy seem to have played a significant role in the differentiation and evolution of these plants. Cytogenetic data (such as banding, fluorescent *in situ* hybridization and genome size assessment) would certainly be of a great interest for understanding the evolution of the family. Nuclear DNA amount assessments constitute a fundamental complement to chromosome counts and, in addition, a powerful tool in order to establish the relationships between closely related taxonomical groups (e.g., in *Echinops* L.; [22], *Orobanche* L.; [23]), as well as to understand the evolution within related genera from a broader point of view (e.g., Liliaceae; [24], Orchidaceae; [25]). Furthermore, genome size is a useful tool to detect possible hybrid and polyploid origins of taxa (e.g., in *Carthamus* L.; [26], *Nicotiana* L.; [27], *Artemisia* L.; [28]), and intraspecific variation can reveal incipient speciation [29].

The aims of the present work are to: (a) enlarge the knowledge concerning nuclear DNA amounts in the Valerianaceae, to date limited to only one report ("*Kentranthus ruber* Druce" 1C = 0.42 pg; [30]), (b) integrate these results with those of chromosome number and molecular phylogeny, and (c) provide a new focus that could contribute, from the perspective of genome evolution, to verify the hypotheses of previous works on the relationships among the above-mentioned genera.

2. Material and Methods

2.1. Plant Material. Table 1 shows the provenance of the species investigated. The sampling includes representatives of all the six genera considered in the family in regard to the results of molecular phylogenies [4–6]. Studied plants come from germinated cypselas, collected in the field or obtained from Botanic Gardens. Due to difficulties in germinating seeds of *Valeriana* representatives, whole individuals were collected in the field and cultivated in the Institut Botànic de Barcelona.

2.2. Karyological and Cytogenetic Analyses

2.2.1. Chromosome Counts and/or Determination of the Ploidy Level. Root tip meristems were obtained either by germinating seeds on wet filter paper in Petri dishes at room temperature, or from plants cultivated in pots in the greenhouse. They were pretreated with 0.002 M 8-hydroxyquinoline for 3 h at 16°C. Material was fixed in absolute ethanol, trichloromethane, and glacial acetic acid (6 : 3 : 1) and stored in the fixative at 4°C during at least two days before processing. Samples were hydrolysed in 1 N HCl for 5 min at 60°C, stained with 1% aqueous aceto-orcein for 1 h minimum, and squashed on slides into a drop of 45% acetic acid-glycerol (9 : 1). Metaphase plates were photographed with a digital camera (Zeiss AxioCam HRm) mounted on a Zeiss Axioplan microscope, and images were analysed with Axio Vision Ac version 4.2.

2.2.2. Nuclear DNA Assessments. Leaf tissue of five individuals for each studied population was chopped in 600 μl of LB01 isolation buffer [33] with a razor blade, together with the chosen internal standard. The species *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg) and *Pisum sativum* L. 'Express long' (2C = 8.37 pg) were used as internal standards [34] to cover the range of 2C-values found. Seeds of the standards were provided by the Institut des Sciences du Végétal (CNRS), Gif-sur-Yvette (France). For each individual, two independent samples were extracted and measured the same day. Samples were supplemented with 100 μg/ml of ribonuclease A (RNase A, Boehringer) and subsequently stained with 36 μl of propidium iodide (1 mg/ml) to a final concentration of 60 μg/ml (Sigma-Aldrich Química), kept on ice for 20 min and measured in an Epics XL flow cytometer (Coulter Corporation). The instrument was set up with the standard configuration. Excitation of the sample was performed using a standard 488-nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC), and red (620 nm) fluorescence for propidium iodide were acquired. Optical alignment was based on optimized

Table 1: Origin of the populations studied with indications of the herbaria where voucher specimens are deposited, chromosome counts, genome size data, and GenBank accession numbers (only for the new sequences).

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Centranthus angustifolius (Mill.) DC. (1)	France: Vaucluse, Mt. Ventoux, Hidalgo 212 & Hidalgo (BC)	P	1.30 ± 0.03	32	4x	0.33	1271.4	3.83 ± 0.63	1.25 ± 0.59	HM162366
Centranthus angustifolius (Mill.) DC. (2)	France: Hautes-Alpes, VI-2005, Hidalgo & Romashchenko (MPU)	P	1.35 ± 0.02	32	4x	0.34	1320.3	5.57 ± 0.86	3.77 ± 0.65	HM162367
Centranthus angustifolius (Mill.) DC. (3)	France: Hautes-Alpes, Les Infournas, Hidalgo 201 & Martin (BC)	P	1.41 ± 0.07	32	4x	0.35	1379	3.35 ± 0.55	1.50 ± 0.43	
Centranthus calcitrapae (L.) Dufr. (1)	Spain, Catalonia, Port d'Àger, Garnatje 229 & Vallès (BCN)	A	1.56 ± 0.04	32	4x	0.39	1525.7	5.22 ± 1.14	4.12 ± 0.95	
Centranthus calcitrapae (L.) Dufr. (2)	France: Gard, Saint André de Majencoules, 4-VI-2005, Hidalgo, Romashchenko & Romo (BC)	A	1.58 ± 0.02	32	4x	0.40	1545.2	3.28 ± 0.70	1.99 ± 0.62	HM162368
Centranthus calcitrapae (L.) Dufr. (3)	Greece, Crete: Elafonissi, Bot. Gard. MNHN 1996-08	A	1.61 ± 0.01	32	4x	0.40	1574.6	3.62 ± 0.96	1.06 ± 0.87	
Centranthus calcitrapae (L.) Dufr. (4)	Greece, Crete: Hania, Bot. Gard. MNHN 1996-15	A	1.62 ± 0.03	32	4x	0.4	1584.4	3.16 ± 1.45	1.51 ± 0.56	
Centranthus cf. *calcitrapae* (L.) Dufr. (5)	Morocco: Hidalgo, Romo 13128 & Soriano (BC)	A	1.80 ± 0.03	**32**	**4*x***	0.45	1760.4	3.46 ± 0.60	2.20 ± 0.70	HM162369
Centranthus lecoqii Jord. (1)	France: Lozère, Les Vignes, Bot. Gard. MNHN 2002-39	P	1.22 ± 0.05	32	4x	0.31	1193.2	3.62 ± 1.58	2.09 ± 0.48	
Centranthus lecoqii Jord. (2)	France: Cons. Bot. Porquerolles Ly815	P		**32**	**4*x***					
Centranthus lecoqii Jord. (3)	France: Hérault, St. Guilhem le Désert, Mathez 1076 (MPU)	P		32	4x					HM162370

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Centranthus longiflorus Steven var. *latifolius* Boiss.	Lebanon: Les Cèdres, Bou Dagher-Kharrat, Hidalgo & Romashchenko 409 (BC)	P	1.42 ± 0.03	32	4x	0.36	1388.8	7.90 ± 0.68	7.71 ± 2.41	
Centranthus macrosiphon Boiss.	Bot. Gard. Hauniensis 2000-2315	A	1.97 ± 0.06	**32**	**4*x***	0.49	1926.7	2.58 ± 1.19	1.64 ± 1.26	HM162371
Centranthus nevadensis Boiss. (1)	Morocco: Akchar, sur Tizi Ouzli, Hidalgo, Romo 13035 & Soriano (BC)	P	1.16 ± 0.02	32	4x	0.29	1134.5	4.23 ± 0.98	2.27 ± 0.77	
Centranthus nevadensis Boiss. (2)	Morocco: Djebel Lechâab, Hidalgo, Romo 13147 & Soriano (BC)	P	1.19 ± 0.01	32	4x	0.3	1163.8	3.80 ± 0.37	1.87 ± 0.78	HM162372
Centranthus ruber (L.) DC. (1)	France: Bouches-du-Rhône, la Sainte Baume, VI-2005, Hidalgo, Romashchenko & Romo (BC)	P	1.14 ± 0.04	32	4x	0.29	1114.9	3.77 ± 1.59	0.81 ± 0.41	
Centranthus ruber (L.) DC. (2)	Rumania: Valea Moril, Distr. Cluj, Bot. Gard. Cluj-Napoca 2006-1820	P	1.14 ± 0.02	32	4x	0.29	1114.9	4.56 ± 0.49	2.36 ± 0.22	HM162373
Centranthus ruber (L.) DC. (3)	Italy, Sicily: Siracusa, Buccheri Mt., Bot. Gard. Palermo 2006-750	P	1.16 ± 0.04	**32**	**4*x***	0.29	1134.5	3.67 ± 0.71	0.88 ± 0.44	
Fedia cf. *cornucopiae* (L.) Gaertn. (1) (as *F. caput-bovis*)	Italy, Sicily: Siracusa, Lauro-Buccheri Mt., Bot. Gard. Palermo 2006-751	A	1.70 ± 0.02	32	4x	0.43	1662.6	2.68 ± 0.31	0.18 ± 0.08	HM162374
Fedia cornucopiae (L.) Gaertn. (2)	Willd coll., Bot. Gard. Coimbra 2005-1321	A	1.79 ± 0.04	32	4x	0.45	1750.6	5.28 ± 0.96	3.28 ± 1.16	
Fedia graciliflora Fisch. & C.A.Mey.	Bot. Gard. Montpellier (MPU)	A	1.53 ± 0.04	32	4x	0.38	1496.3	4.48 ± 0.96	2.53 ± 1.17	HM162375

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	2n[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Fedia pallescens (Maire) Mathez	Morocco: Mehdyia, El-Oualidi s.n.-1998 (MPU)	A	1.14 ± 0.02	32	4x	0.29	1114.9	5.85 ± 1.54	2.71 ± 1.14	HM162376
Nardostachys jatamansi (D. Don) DC.	Bot. Gard. Lautaret, France	P	3.49 ± 0.04					2.06 ± 0.72	1.25 ± 0.82	
Patrinia rupestris Dufr.	Russia: Siberia, Bot. Gard. Lautaret, France	P	2.56 ± 0.01					2.81 ± 1.01	1.87 ± 1.1	
Patrinia scabiosifolia Link (1)	Japan: Bot. Gard. Ofuna 314-2006	P	1.50 ± 0.03					5.09 ± 0.71	4.08 ± 0.37	
Patrinia scabiosifolia Link (2)	Japan: Tsukiono-machi, Tone-gun, Bot. Gard. Chiba University 2006-193	P	2.57 ± 0.07	**ca. 44**	**4*x***	0.64	2513.5	3.7 ± 0.68	1.91 ± 0.58	HM162377
Valeriana apula Pourr.	Spain, Aragon: Huesca, Anglios, 2005, Vallès (BCN)	P	0.97 ± 0.02	**16**	**2*x***	0.49	948.66	5.58 ± 0.87	2.16 ± 0.54	HM162378
Valeriana celtica L. (1)	France: Savoie, Evettes circus, Bot. Gard. Lautaret, France	P	2.11 ± 0.04					1.81 ± 1.37	1.29 ± 1.10	
Valeriana celtica L. (2)	Italy: Aosta, Cogne, Hidalgo 503 (MPU)	P								HM162379
Valeriana dioica L. (1)	France: Hautes-Alpes, Lautaret pass, VI-2005 Douzet, Hidalgo & Romashchenko (BC)	P	2.87 ± 0.05					3.25 ± 0.31	0.72 ± 0.70	
Valeriana dioica L. (2)	France: Gard, Bonheur valley, 5-VI-2005, Hidalgo, Romashchenko & Romo (BC)	P	3.01 ± 0.01 (♀) 3.08 ± 0.01 (♂)					3.11 ± 0.21 (♀) 3.22 ± 0.14 (♂)	0.72 ± 0.49 (♀) 1.78 ± 0.51 (♂)	HM162380
Valeriana longiflora Willk. (1)	Spain, Aragon: Santa Anna dam, Garnatje 172 & Vallès (BCN)	P	0.96 ± 0.02					5.84 ± 0.99	2.91 ± 0.30	

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx[4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Valeriana longiflora Willk. (2)	Spain, Aragon, Santa Anna dam, Garnatje, Hidalgo 500 & Luque (MPU)	P								HM162381
Valeriana montana L. (1)	France: Hautes-Alpes, Les Infournas, Hidalgo 202 & Martin (BC)	P	1.23 ± 0.01					3.92 ± 1.32	1.59 ± 1.05	
Valeriana montana L. (2)	Spain, Catalonia: Girona, Camprodon, Garnatje 179 (BC)	P	2.24 ± 0.04					5.98 ± 2.41	0.88 ± 0.62	
Valeriana montana L. (3)	Italy: Maritime Alps, Cuneo, 6-VIII-2005, Peccenini & Vallès (BCN)	P	2.40 ± 0.05					2.79 ± 0.36	2.21 ± 0.83	
Valeriana montana L. (4)	France: Hautes-Alpes, between the Galibier and Lautaret passes, Garnatje & Hidalgo 220 (BC)	P	2.42 ± 0.03					2.79 ± 0.49	2.17 ± 0.73	HM162382
Valeriana montana L. (5)	Spain, Catalonia: Lleida, Vall d'Aran, on the way to the Restanca from Arties, Garnatje 234 & Vallès (BCN)	P	2.50 ± 0.05	**32**	***4x***	0.63	2445	2.08 ± 0.28	1.32 ± 0.80	HM162383
Valeriana montana L. (6)	France: Hautes-Alpes, spontaneous in the Lautaret Bot. Gard., Garnatje & Hidalgo 221 (BC)	P	2.51 ± 0.03					2.81 ± 0.69	2.35 ± 0.84	
Valeriana montana L. (7)	Spain, Catalonia: Lleida, Vall d'Aran, on the way to the Restanca from Arties, Garnatje 235 & Vallès (BCN)	P	2.54 ± 0.03					2.52 ± 0.98	0.87 ± 0.77	HM162384
Valeriana montana L. (8)	France: Hautes-Alpes, between Le Roy and Pise pass, Hidalgo 210 & Martin (BC)	P	2.60 ± 0.03	**32**	***4x***	0.65	2542.8	2.38 ± 0.83	1.36 ± 0.54	

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Valeriana montana L. (9)	France: Hautes-Alpes, Gleize pass, Hidalgo 208 & Martin (BC)	P	2.64 ± 0.05					2.48 ± 1.00	1.82 ± 0.70	HM162385
Valeriana montana L. (10)	France: Hautes-Alpes, Galibier pass, 4-VIII-2001, Hidalgo & Hidalgo (BC)	P								HM162386
Valeriana cf. *montana* L. (11)	Spain, Catalonia: Barcelona, Montserrat, 2005, Casanova (BC)	P	2.46 ± 0.03					3.65 ± 2.06	2.06 ± 0.67	
Valeriana cf. *montana* L. (12)	France: Hautes-Alpes, Gleize pass, VI-2005, Hidalgo & Romashchenko (BC)	P	2.54 ± 0.02					3.3 ± 0.24	1.98 ± 0.60	
Valeriana officinalis L. (1)	Croatia: Dalmatia, Biokovo Mt., 2006, Siljak-Yakovlev (BC)	P	2.97 ± 0.04	**14**	***2x***	1.49	2904.7	4.07 ± 0.29	2.22 ± 0.49	HM162387
Valeriana officinalis L. (2)	France: Gard, Aigoual Mt., 3-VI-2005, Hidalgo, Romashchenko & Romo (BC)	P	3.16 ± 0.03					3.19 ± 0.24	1.44 ± 0.42	HM162388
Valeriana officinalis L. (3)	Spain, Catalonia: Girona, Llanars, Garnatje 169, D. Roca & J. Roca (BC)	P	4.62 ± 0.08					2.76 ± 0.48	3.60 ± 0.66	
Valeriana officinalis L. (4)	Spain, Catalonia: Lleida, Vielha, 2005, Vallès (BCN)	P	6.51 ± 0.11					1.49 ± 1.01	2.89 ± 1.24	
Valeriana officinalis L. (5)	Spain, Catalonia: Lleida, Vall d'Aran, Garnatje 175 & Vallès (BCN)	P	8.05 ± 0.08					1.78 ± 0.76	2.91 ± 0.64	

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Valeriana officinalis L. (6)	Norway: Trs, Storfjord, Rovijok, Bot. Gard. Oulu 2004/5-425	P	8.15 ± 0.25	**ca. 56**	**8*x***	1.02	7970.7	1.40 ± 0.92	2.52 ± 1.67	HM162389
Valeriana officinalis L. (7)	France: Gironde, Gradignan, V-2005, Hidalgo & Revel-Hidalgo (BC)	P	8.32 ± 0.10 (leaf) 8.09 ± 0.08 (root)					3.14 ± 1.26 (leaf) 1.40 ± 1.11 (root)	4.99 ± 0.49 (leaf) 5.83 ± 0.47 (root)	
Valeriana pyrenaica L.	Spain, Catalonia: Lleida, Vall d'Aran, Garnatje 176 & Vallès (BCN)	P	1.26 ± 0.01	**16**	**2*x***	0,6	1232.3	5.37 ± 0.64	2.81 ± 0.26	HM162390
Valeriana rotundifolia Vill.	France: Hautes-Alpes, Devoluy, La Cluse, Hidalgo 207 & Martin (BC)	P	2.52 ± 0.05					2.16 ± 0.30	1.41 ± 0.51	HM162391
Valeriana saliunca All.	France: Savoie, Galibier pass, Garnatje 166 & Vallès (BC)	P	1.46 ± 0.03	**16**	**2*x***	0.73	1427.9	4.21 ± 0.36	3.21 ± 0.25	HM162392
Valeriana saxatilis L. (1)	Italy: Apuan Alps, summit of Sagro Mt., Hidalgo 213 (BC)	P	1.05 ± 0.00					3.17 ± 0.28	2.21 ± 0.65	
Valeriana saxatilis L. (2)	Italy: Apuan Alps, Hidalgo 217 (BC)	P	1.02 ± 0.01					3.62 ± 0.38	2.36 ± 0.24	HM162393
Valeriana tripteris L. (1)	France: Hautes-Alpes, Lautaret pass, Hidalgo 203 & Martin (BC)	P	1.46 ± 0.03	**16**	**2*x***	0.73	1427.9	3.28 ± 0.71	1.79 ± 0.69	
Valeriana tripteris L. (2)	France: Gard, Mt. Aigoual, 3-VI-2005, Hidalgo, Romashchenko & Romo (BC)	P	1.51 ± 0.01 (♀♂) 1.52 ± 0.02 (♀)					4.01 ± 0.38 (♀♂) 3.75 ± 0.24 (♀)	2.24 ± 0.48 (♀♂) 2.12 ± 0.53 (♀)	
Valeriana tripteris L. (3)	France: Vaucluse, Mt. Ventoux, Hidalgo 211 & Hidalgo (BC)	P	1.51 ± 0.01	**18**	**2*x***	0.76	1476.8	2.75 ± 0.35	1.34 ± 0.40	HM162394

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Valeriana tripteris L. (4)	Spain, Catalonia: Lleida, Espot, road to Espot Ski, 17-VI-2007, Garnatje 233 & Vallès (BCN)	P	1.54 ± 0.03					5.63 ± 1.00	4.86 ± 1.03	HM162395
Valeriana tripteris L. (5)	Spain, Catalonia: Girona, Setcases, Garnatje 170, D. Roca & J. Roca (BC)	P	1.58 ± 0.06					3.88 ± 0.74	2.01 ± 0.81	HM162396
Valeriana tripteris L. (6)	France: Gard, Mt. Aigoual, 14-VII-00, Hidalgo (BC)	P								HM162397
Valeriana cf. *tripteris* L. (7)	Italy: Apuan Alps, Hidalgo 218 (BC)	P	1.23 ± 0.06	**32**	**4*x***	0.31	1202.9	3.93 ± 0.66	2.34 ± 0.98	HM162398
Valeriana tuberosa L.	France: Les Sièges, Mathez 1088 (MPU)	P	3.74 ± 0.03					2.96 ± 0.99	3.66 ± 0.42	HM162399
Valerianella coronata (L.) DC. (1)	Italy: Aymavilles, Cerignan, Bot. Gard. Cogne 2006/7-838	A	0.48 ± 0.00	**14**	**2*x***	0.24	469.44	0.59 ± 0.23	0.99 ± 0.53	
Valerianella coronata (L.) DC. (2) (as *V. pumila*)	France: Vaucluse, "la Bastide des Jourdans", Cons. Bot. de Porquerolles 1998-CO 2688	A	0.53 ± 0.01	**14**	**2*x***	0.27	518.34	8.90 ± 0.70	3.26 ± 0.65	
Valerianella dentata (L.) Pollich (1) (as *V. rimosa*)	France: Maine-et-Loire, Montreuil-Bellay, "Méron", Bot. Gard. Nantes 2004-306	A	0.42 ± 0.01	**16**	**2*x***	0.21	410.76	11.88 ± 2.54	0.55 ± 0.37	

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Valerianella dentata (L.) Pollich (2)	Bot. Gard. Bonn 2004/5-23687	A	0.49 ± 0.01 (leaf) 0.47 ± 0.02 (root)	**16**	**2*x***	0.25	479.02	9.64 ± 0.85 (leaf) 8.69 ± 0.83 (root)	1.09 ± 0.85 (leaf) 2.29 ± 0.74 (root)	
Valerianella dentata (L.) Pollich (3)	Italy, Sicily: Palermo, Rocca Busambra-Ficuzza, Bot. Gard. Palermo 2006-756	A		16	2*x*					HM162400
Valerianella discoidea Loisel	Greece, Crete: Hania, Bot. Gard. MNHN 2005-96/14	A		**14**	**2*x***					
Valerianella echinata DC.	France: Aveyron, near Brunas de Creissels (Larzac N.), Bernard	A	0.60 ± 0.01	16	2*x*	0.3	586.8	5.54 ± 2.24	2.16 ± 0.71	
Valerianella eriocarpa Desv. (1)	Italy, Sicily: Palermo, Rocca Busambra-Ficuzza, Bot. Gard. Palermo 2006-757	A	0.47 ± 0.02	**16**	**2*x***	0.24	459.66	6.31 ± 0.50	0.92 ± 0.86	HM162401
Valerianella eriocarpa Desv. (2)	Bot. Gard. Nantes 2004-302	A	0.54 ± 0.01	16	2*x*	0.29	528.12	10.07 ± 2.83	1.86 ± 0.61	
Valerianella locusta L. (1)	France: Loire Atlantique, cheméré "Le Moulin", Bot. Gard. Nantes 2004-303	A	0.44 ± 0.02	**16**	**2*x***	0.22	430.32	11.37 ± 1.69	1.18 ± 0.84	HM162402
Valerianella locusta L. (2) (as *V. carinata*)	France: Loire Atlantique, Gard. Nantes 2004-301	A	0.49 ± 0.02	16	2*x*	0.25	479.22	8.50 ± 1.57	0.20 ± 0.19	HM162403

Table 1: Continued.

Species (population number)	Collection data	Life cycle[1]	2C (pg)[2]	$2n$[3]	Ploidy level	1Cx [4]	2C (Mbp)[5]	Mean HPCV of sample (%)	Mean HPCV of standard (%)	*trn*L-*trn*F GenBank accession number
Valerianella microcarpa Loisel	Spain, Catalonia: Tarragona, Ebre delta, 2004, Pyke (BC)	A	0.46 ± 0.02	16	2x	0.23	449.88	8.43 ± 1.29	0.22 ± 0.20	
Valerianella multidentata Loscos & Pardo	Spain, Aragon: Zaragoza, 2004, Pyke (BC)	A	0.52 ± 0.02	**14–16**	**2*x***	0.26	508.56	7.78 ± 0.77	0.97 ± 0.92	HM162404
Valerianella pontica Velen.	Ukraine: Crimea, Boratinsky, Didukh, Romashchenko, Romo 1029bis & Susanna (BC)	A	0.51 ± 0.02		2x	0.26	498.78	11.47 ± 1.33	0.18 ± 0.10	
Valerianella turgida Betcke (1)	Greece: Oxia, Bot. Gard. MNHN 2002-190	A	0.39 ± 0.01	**16**	**2*x***	0.2	381.42	12.15 ± 1.54	0.65 ± 0.67	HM162405
Valerianella turgida Betcke (2)	Ukraine: Crimea, Boratinsky, Didukh, Romashchenko, Romo 10171 & Susanna (BC)	A	0.47 ± 0.02	16	2x	0.24	459.66	7.49 ± 2.66	1.97 ± 0.52	HM162406
Valerianella vesicaria Moench	Israel: Judean Mts. Bot. Gard. Tel Aviv 2001	A	0.61 ± 0.05		2x	0.31	596.58	9.58 ± 2.98	0.36 ± 0.33	

[1] Life cycle: A annual, P perennial. [2] 2C nuclear DNA content (means ± SD of 10 measurements—two replicates for five individuals each). [3] $2n$ somatic chromosome number. $2n$ and ploidy levels are estimated through bibliographical record (using the database http://www.tropicos.org/), except those in bold, which were verified in this study. [4] 1Cx monoploid genome size (DNA content per basic chromosome set [31]). [5] 2C [Mbp]: 1 pg = 978 Mbp [32].

signal from 10-nm fluorescent beads (Immunocheck, Epics Division, Coulter Corporation). Time was used as a control for the stability of the instrument. Red fluorescence was projected onto a 1,024 monoparametric histogram. Gating single cells by their area versus peak fluorescence signal excluded aggregates. Acquisition was automatically stopped at 8,000 nuclei. Measurements were made at the Serveis Cientificotècnics (Universitat de Barcelona).

2.2.3. Effect of Valepotriates on Measurements. Valerianaceae contain, *inter alia*, valepotriates, a family of chemical compounds of great medicinal interest, thanks to which some representatives are considerably exploited by the pharmaceutical industry. Valepotriates are known DNA intercalators (they do have severe effects on DNA in e.g., PCR amplifications) and might thus possibly influence genome size measurements by altering the hydrodynamic diameter of the DNA. Furthermore, they could constitute endogenous staining inhibitors, which affect results by causing stoichiometric errors [35, and references therein]. Valepotriates are mainly stored in oil vesicles in the roots and rhizomes [36]. Therefore, we performed measurements on some roots and compared them with DNA C-values obtained for leaves of the same individuals to detect a potential effect of these chemical compounds on genome size assessments if any.

2.2.4. Statistical Analyses. ANOVA and LSD test were performed with the Statgraphics Plus 5.1 program (Statistical Graphics Corp., USA).

2.3. Phylogenetic Analyses

2.3.1. DNA Extraction, Amplification and Sequencing. Total genomic DNA was extracted from silica-dried, herbarium voucher or fresh leaves using the Nucleospin Plant extraction kit (Macherey-Nagel, GmbH & Co., Duren, Germany). PCRs were carried out with PTC100 (MJ Research, Inc.) research thermal cyclers in 25 μl volume. The whole plastid *trn*L-*trn*F region [including the *trn*L intron, the 3′*trn*L (UAA) exon, and the intergenic spacer between *trn*L (UAA) and *trn*F (GAA)] was amplified and sequenced with the universal primers trnL-c, forward, and trnL-f, reverse, and, in some cases, trnL-d, reverse, and trnL-e, forward [37]. The PCR amplification conditions used were 94°C, 1 min 35 sec; 34x (93°C, 1 min; 58°C, 1 min; 72°C, 1 min); 72°C, 10 min, and storage at 4°C. PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) or with DNA Clean & Concentrator-5 D4003 (Zymo Research, Orange, California, USA). Direct sequencing of the amplified fragments was performed using the Big Dye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, California, USA). Nucleotide sequencing was carried out at the Serveis Cientificotècnics (Universitat de Barcelona) with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Foster City, California, USA).

2.3.2. Sequence Assembly, Alignment, and Analyses. Nucleotide sequences were assembled and edited using MacClade 4.08 [38]. Bayesian inference (BI) was carried out with MrBayes version 3.1.2 [39]. The most appropriate nucleotide substitution models were chosen with MrModeltest version 2.3 [40]. Four Markov chains were run simultaneously for 1×10^6 generations, and these were sampled every 100 generations. Data from the first 1,000 generations were discarded as the *burn-in* period, after confirming that likelihood values were stabilized prior to the 1,000th generation. The 50% majority rule consensus trees and posterior probability (PP) of nodes were calculated from the pooled samples. We proceeded to an additional analysis, adding to the matrix the gap data codified with the Barriel method, as previously used for Valerianaceae in [3]. Partitioned dataset analysis was carried out for this dataset including the codified gaps. A gamma-shaped rate variation was stated for the codified data following the manufacturer's protocol, applying the model selected with MrModeltest for the DNA data.

3. Results

3.1. Chromosome Counts. Chromosome numbers of the populations studied are indicated in Table 1, and metaphase plates are presented in Figure 1. According to our data, we provide the first count for *Valerianella multidentata* Loscos & Pardo. Ploidy levels ranging from diploid to octoploid were detected although diploid and tetraploid were predominantly reported. Good metaphase plates are quite difficult to obtain for Valerianaceae. In fact, this is certainly the main reason why only few chromosome studies have been carried out in the family, and that especially few chromosome pictures have been published to present.

3.2. Genome Size Assessments. Data on nuclear DNA content are presented in Figure 2 and Table 1. The present data are the first reports on nuclear DNA content in all the genera studied excepting *Centranthus* and in all the species studied of this genus except for *C. ruber* (L.) DC., which was to date the only member of the family with a known genome size [30]. Genome size in Valerianaceae varies about 21.3-fold, from 2C = 0.39 pg (*Valerianella turgida* Betcke, 2*x*) to 2C = 8.32 pg (*Valeriana officinalis*, 8*x*). Although the half peak coefficients of variation (HPCV) are rather high for some species (in all cases but one of the genus *Valerianella*), their mean value is 4.62% for the target plants and 2.04% for the standards. More in-deep research is suitable in *Valerianella* to try and obtain measurements with better HPCV.

The difference in genome size between leaves and roots measured in the same population has been addressed for *Valeriana officinalis* (7) (leaves: 2C of 7.67–8.97 pg and roots: 2C of 8.04–8.14 pg, at 95% confidence interval) and *Valerianella dentata* (L.) Pollich (2) (leaves: 2C of 0.485–0.495 pg and roots: 2C of 0.464–0.476 pg, at 95% confidence interval). A significant difference has been detected for *Valerianella dentata* (ANOVA, $P = 0.0036$), which may indicate a possible effect of valepotriates on genome size assessments in this species, also suggested by the especially high HPCV values found in this annual genus (Table 1).

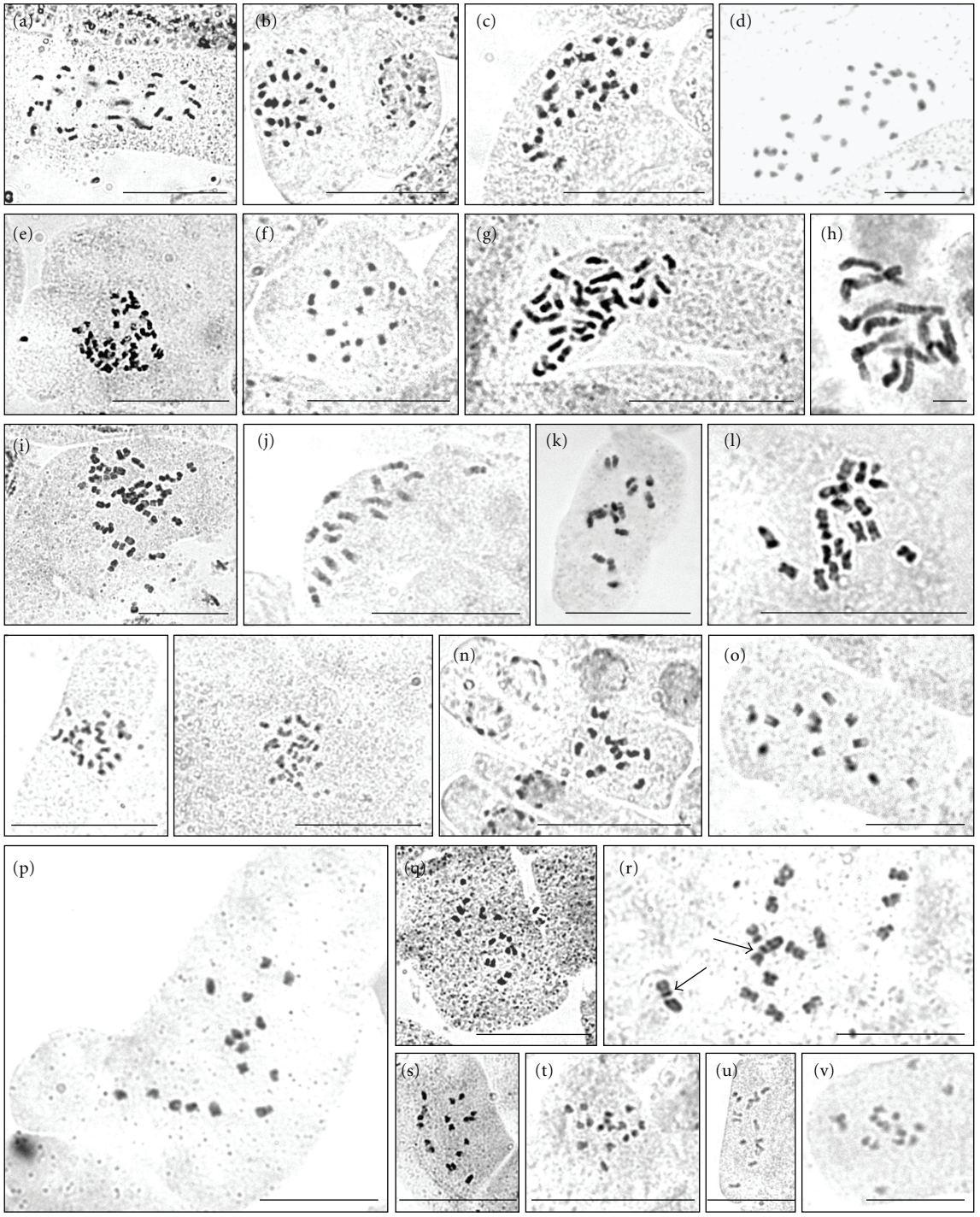

Figure 1: (a)–(v). Somatic metaphases. (a) *Centranthus* cf. *calcitrapae* (5), $2n = 32$. (b) *C. lecoqii* (2), $2n = 32$. (c) *C. macrosiphon*, $2n = 32$. (d) *C. ruber* (3), $2n = 32$. (e) *Patrinia scabiosifolia*, $2n$= ca. 44. (f) *Valeriana apula* $2n = 16$. (g) *V. montana* (5), $2n = 32$. (h) *V. officinalis* (1), $2n = 14$. (i) *V. officinalis* (6), $2n$ = ca. 56. (j) *V. pyrenaica*, $2n = 16$. (k) *V. saliunca,* $2n = 16$. (l) *V. tripteris* (3), $2n = 18$. (ma-b) *V.* cf. *tripteris* (7), $2n = 32$. (n) *Valerianella coronata* (1), $2n = 14$. (o) *V. coronata* (2), $2n = 14$. (p) *V. dentata* (1), $2n = 16$. (q) *V. dentata* (2), $2n = 16$. (r) *V. discoidea*, $2n = 14$. Arrows indicate a chromosome pair much larger than the rest. (s) *V. eriocarpa* (1), $2n = 16$. (t) *V. locusta*, $2n = 16$. (u) *V. multidentata*, $2n = 14$–16. (v) *V. turgida* (1), $2n = 16$. Scale bars = 10 μm.

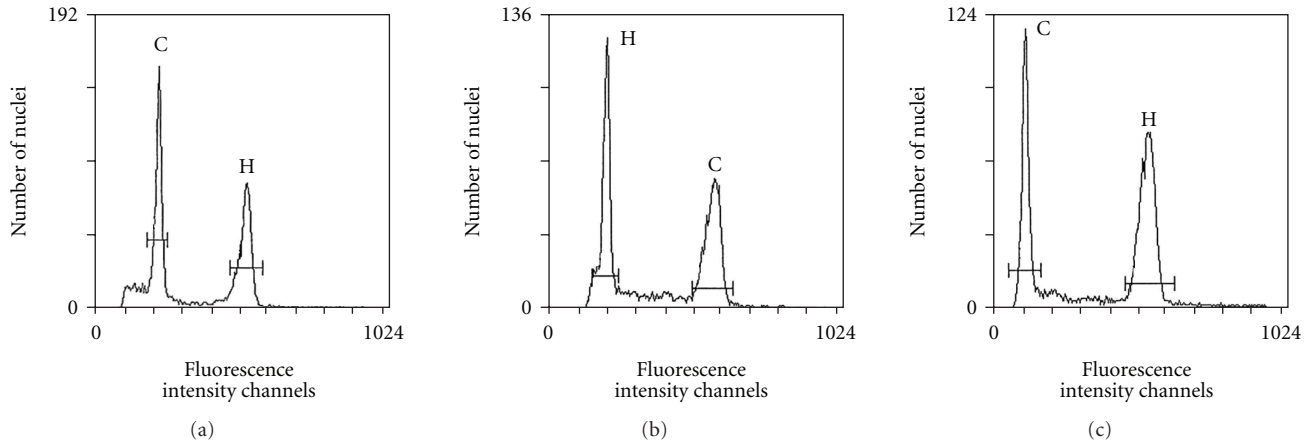

Figure 2: (a)–(c). Histograms of nuclear DNA content obtained for some representatives of Valerianaceae. (a) *Centranthus nevadensis* (2) (2C = 1.19 ± 0.01). (b) *Valeriana officinalis* (7) (2C = 8.32 ± 0.10). (c) *Valerianella coronata* (2) (2C = 0.53 ± 0.01). Peak C: sample species nuclei. Peak H: standard species nuclei.

3.3. Molecular Phylogeny. Both AIC (Akaike Information Criterion) and hLRT (hierarchical Likelihood Ratio Tests) selected GTR+G (General Time Reversible model with gamma distribution) as the best-fit model. The results of the Bayesian analyses are presented in Figure 3. Phylogenetic trees obtained through the analyses of the two different datasets basically led to the same tree topology and supports. Nevertheless, three clades are only significantly supported when codified gap information is taken into account. These are (a) *Valeriana* clade I (PP = 0.95, Figure 3), (b) *Valeriana longiflora* Willk. plus *Centranthus* clade (PP = 1.00, Figure 3), and (c) the grouping of *Fedia cornucopiae* (L.) Gaertn. AF446986 with *Fedia graciliflora* Fisch. & C.A.Mey. (PP = 0.96, Figure 3).

4. Discussion

We address here the analysis of genome size variation and its phylogenetic and taxonomical implications in the taxa considered.

4.1. The Basal Grades. They consist of the taxa showing the most ancestral characters within the Valerianaceae, namely, the genera *Nardostachys* and *Patrinia*, along with the clade grouping *Valeriana celtica* and *Valeriana saxatilis* L. (*Valeriana* clade III, Figure 3). Shifts in biogeography, flower morphology, and basic chromosome number occurred at this point of the evolutionary history of the family. From this moment on, the distribution area of the Valerianaceae, until that time restricted to Asia (*Nardostachys* and *Patrinia*), was enlarged to the other continents. At the same time the number of stamens decreased from four to three. These changes just preceded the change in basic chromosome numbers from $x = 11$ to $x = 8$, occurring at the arising of Valerianeae tribe (Figure 3).

The *Valeriana* clade III (P = 1.00, Figure 3) constituted by two species from the Alps, *V. celtica* and *V. saxatilis*, is branched between Patrinieae and Valerianeae, a position that was previously found for *V. celtica* [4, 5], and stated for the first time here for *V. saxatilis*. Although their genome sizes fall within the range of the remaining *Valeriana*, these two valerian species present $x = 11$ as basic chromosome number [41, 42], which had only been detected in *Patrinia*. Furthermore, *V. celtica* has a yellow corolla, also like species of *Patrinia*. Nevertheless, species of clade III differ from *Patrinia* by a pappus-like *Centranthus* and *Valeriana*, and the number of stamens reduced to three as in *Valeriana* and *Valerianella* (whereas *Nardostachys* presents four and occasionally five stamens and *Patrinia* presents five or less stamens; [43, 44]). Therefore, species of clade III are from morphological, cytogenetic, and phylogenetic points of view in between Patrinieae and Valerianeae. These results raise numerous questions. Should this clade III form a new genus? Could any other *Valeriana* species be susceptible to join this group? Then, should this group be classified within Valerianeae, or as a new tribe (their inclusion in the Patrinieae being impossible under the monophyly criterion)?

4.2. Fedia *Plus* Valerianella *Clade.* These species have the smallest genome sizes known in the family (Table 1) to present. *Fedia* and *Valerianella* are exclusively composed of annual herbaceous plants. All the species belonging to these two genera, excepted *F. pallescens* (Maire) Mathez, are common weeds in waste ground and cultivated land. *Valerianella*, which comprises around 50 species that are mainly diploid and with three stamens, is widely distributed in Eurasia, Africa and America. The genus *Fedia* includes only three tetraploid species and one hexaploid subspecies [45], with two stamens, and is restricted to the Mediterranean region. Molecular inferences suggest that *Fedia* species should have originated from *Valerianella* (Figure 3; [7]), in other words that autogamous strongly associate to anthropogenic environment (*Valerianella*) would have given rise to allogamous, polyploid (*Fedia*).

Genome size in those genera shows that *Fedia* ($1Cx$ = 0.29 to 0.45 pg) has significantly higher DNA values than

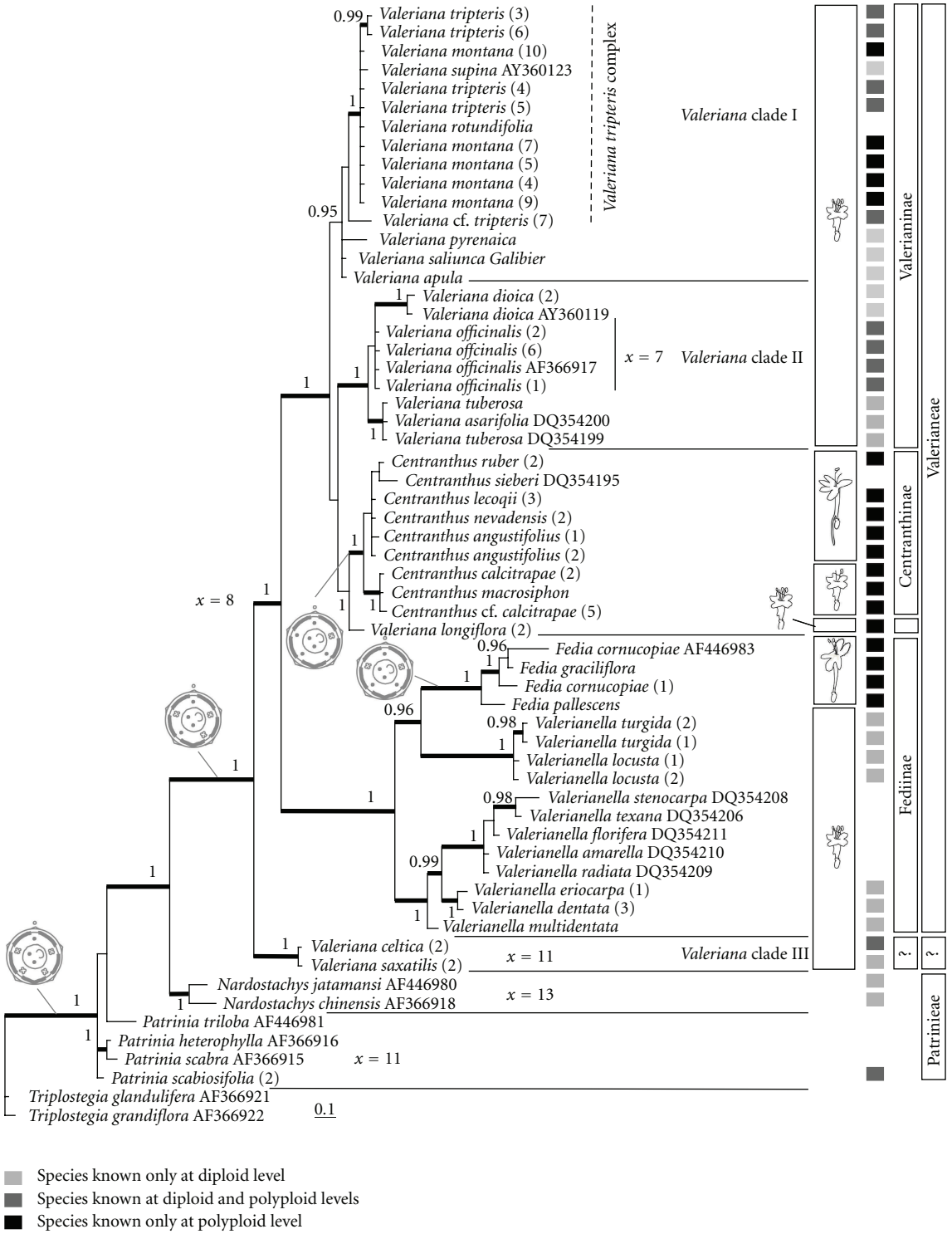

Figure 3: Molecular phylogeny based on the Bayesian analysis of the *trn*L-*trn*F region and the codified gap information, with posterior probability values ≥0.95 indicated on the branches. Wider branches are those significantly supported in the Bayesian analysis of the *trn*L-*trn*F region without including the information from the gaps. Taxonomic information, relevant floral characters, basic chromosome number, and data on ploidy levels are indicated. GenBank accession numbers are provided for the previously published sequences. Numbers in brackets differentiate populations of a same species (see Table 1).

Valerianella (from 1Cx = 0.20 to 0.31 pg) (P = 0.0000), which corroborates the conclusions of Albach & Greilhuber [46] that allogamy is related to higher DNA values than autogamy. Furthermore, *Fedia* presents the "allogamy syndrome," morphological adaptations linked to the breeding system (e.g., zygomorphic corolla, development of a nectar gibbosity, tube elongation, and occurrence of polychroic corolla). The intensity of this syndrome varies between the species and the subspecies of *Fedia* [47]. The tendency within the genus is toward an increasing genome size and degree of allogamy syndrome, from *Fedia pallescens* (1Cx = 0.29 pg) to *F. graciliflora* (1Cx = 0.38 pg) and *F. cornucopiae* (1Cx = 0.43–0.45 pg).

The *Valerianella* group splits in two major clades (Figure 3), one including *V. turgida*, *V. locusta* (L.) Laterr., and the *Fedia* species. The genus *Valerianella* is revealed in this study as a relatively homogeneous group respecting genome size within Valerianaceae, with 2C values that range from 0.39 pg (*V. turgida*) to 0.61 pg (*V. vesicaria* Moench). Furthermore, some of *Valerianella* species, such as *V. turgida*, account for very small genomes, which fall into the range of the smallest genome size records in angiosperms [48], around the same scale than *Arabidopsis thaliana* (L.) Heynh. (1C = 0.16 pg; [49]), for long considered as the lowest angiosperms C-DNA value, nowadays reported in the genus *Genlisea* (*G. margaretae* 1C = 0.064 pg; [50]).

The chromosome number of *Valerianella multidentata*, $2n = 2x$ = 14–16, is reported here for the first time. This endemic restricted to a small area of Catalonia, Spain, is very close to *V. discoidea* Loisel (see [51]; $2n = 2x$ = 14–16), which has been in turn related to *V. coronata* (L.) DC. ([52]; $2n = 2x = 14$). All these species belong to section *Coronatae*. The chromosome number of $2n = 14$ is the smaller within the genus. Metaphase plates of *V. discoidea* (Figure 1(r)) show a chromosome pair much larger than others that may result from a chromosome fusion sparking off the descending diploidy observed in the section.

4.3. Centranthus *Plus* Valeriana longiflora *Clade.* *Centranthus* is a circum-Mediterranean genus of nine species, characterized by flowers with one stamen, related to *Valeriana longiflora* (PP = 1.00, Figure 3; [5]). All the species show the same chromosome number, $2n = 4x = 32$, both annuals (*C. calcitrapae* (L.) Dufr., *C. macrosiphon* Boiss.) and perennials (the other species), and both widespread (*C. calcitrapae*, *C. ruber*) and narrowly distributed species (the remaining). *Centranthus* is organized in three sections: Section *Calcitrapae* (*C. calcitrapae* and *C. macrosiphon*), sect. *Centranthus* (represented in our study by *C. angustifolius* (Mill.) DC., *C. lecoqii* Jord., *C. longiflorus* Stev. and *C. ruber*), and sect. *Nervosa* (not represented in our study). In this case, section *Calcitrapae*, annual (1Cx = 0.39–0.49 pg), has higher genome size than section *Centranthus*, perennial (1Cx = 0.29–0.36 pg). Annual plants have been usually reported to account for smaller genome sizes than perennials [53], as it is comprehensible that the transcriptional machinery would be more efficient in smaller genomes, in order to quickly complete the life-cycle. Otherwise, exceptions to this rule have been found in different plant groups [22, 54], and this seems to be the trend in the genus *Centranthus*.

The common occurrence of hybridization events in the overlapping areas of *Centranthus* species [55], and the fact that some species or infraspecific taxa show intermediate morphological characters, can indicate that several of these taxa could have a hybrid origin. As it has been reported in different plant groups, nuclear DNA content of hybrids corresponds to approximately the mean of both parental genome sizes, or is slightly smaller/larger than expected [26, 27, 56–64]. Within the section *Calcitrapae*, representatives of southern Spain and Morocco have a doubtful taxonomic assignation, somewhere between *C. calcitrapae* and *C. macrosiphon* ([55] for southern Spain; e.g., J Mathez, Université Montpellier II, France, "unpubl. res." for Morocco). One population of Morocco was assessed, *C.* cf. *calcitrapae* (5), and the 1Cx value obtained of 0.45 pg turns out to be the average between the means of typical *C. calcitrapae* (1Cx = 0.40 pg) and *C. macrosiphon* (1Cx = 0.49 pg), this being consistent with the hypothesis of a hybrid origin for this Moroccan population. Also, *C. lecoqii* is morphologically intermediate between *C. angustifolius* and *C. ruber* [55]. The 1Cx amount of *C. lecoqii* (0.31 pg), the mean of those of *C. angustifolius* (0.34 pg) and *C. ruber* (0.29 pg), is compatible with the hypothesis of a hybrid origin for the former taxon.

4.4. Valeriana montana *Clade.* This group of alpine plants is constituted by species with hermaphrodite flowers and of restricted area (*V. saliunca* All. and *V. supina* Ard. from the Alps, *V. pyrenaica* L. and *V. apula* Pourr. from the Pyrenees), two gynodioecious species widely distributed, *V. tripteris* L. and *V. montana* L. (from the mountains of Southern Europe). *Valeriana rotundifolia* Vill. is a gynodioecious taxon of obscure status, present in the Alps and Corsica, and morphologically close to *V. tripteris* and *V. montana* [65]. The molecular phylogeny shows a *Valeriana tripteris* complex constituted by the populations of *V. montana*, *V. rotundifolia*, *V. supina* and *V. tripteris*, who are forming a robust clade (PP = 1.00, Figure 3), with the exception of *V.* cf. *tripteris* (7) that is sister to the remaining ones. This result raises the question of the monophyly of *V. tripteris*, but also shows a possible interesting biogeographical pattern as the population *V.* cf. *tripteris* (7) is the only sequenced that grows at the east of the Alps. Furthermore, this population is not only divergent in terms of DNA sequence, but also for its cytogenetic characters. Although *V.* cf. *tripteris* (7) is a tetraploid (Figure 1 ma-b), its genome size 2C = 1.23 pg ranks low if compared with tetraploid *V. montana* (2C = 2.50–2.60 pg), and even with diploid *V. tripteris* (2C = 1.46–1.51 pg).

Intermediate forms between *V. tripteris* and *V. montana*, such as V. cf. *montana* (11-12), *V.* cf. *tripteris* (7) or *V. rotundifolia*, occur throughout the range of distribution, which makes their taxonomic delimitation difficult. This is the reason why these taxa have been sometimes considered to represent a single species with various subspecific entities, and also as two closely related species with their intermediate

forms as subspecies (see [65], and references therein). Cryptic hybrid and/or polyploid taxa may largely account for the taxonomic heterogeneity of *V. tripteris* complex and the genome size variation observed in the group, especially at intraspecific level. However, Briquet & Cavillier [65] pointed out that intermediate forms are found in isolated populations, without *V. montana*, which makes their hybrid origin improbable and their polyploid origin from *V. tripteris* maybe more likely. Should this polyploid hypothesis be sustained, the question remains as to why tetraploid *V. tripteris* are morphologically similar to *V. montana*. One explanation could be that the speciation of *V. montana* could have also been induced by a polyploidization event of *V. tripteris*, which would have arisen previously in evolutionary time. This would explain both the more notable morphological and ecological differentiation between the two species, and in some cases the more important decrease in monoploid genome size with respect to that of the intermediate forms [23].

4.5. Valeriana officinalis *Clade (*Valeriana *Clade II, Figure 3).* *Valeriana officinalis* and relatives constitute a difficult complex of numerous taxa of specific and infraspecific ranges, with doubtful morphological delimitation. *Valeriana officinalis* presents an unusual basic chromosome number for the genus of $x = 7$. This basic number is also found in *V. wallrothii* Kreyer, a species closely related to *Valeriana officinalis* in the molecular phylogeny [6], but not in other representatives of the group as, for example, *V. dioica* or *V. tuberosa*. The populations of *V. officinalis* measured exhibit different DNA amounts, suggesting four different DNA ploidy levels of 2*x*, 4*x*, 6*x*, and 8*x* (Table 1). Even so, it would be necessary to determine whether those differences really correspond to different ploidy levels, or if they are partially due to a high intraspecific variability within *V. officinalis*. This doubt concerns particularly the ploidy levels that are uncommon in the *V. officinalis* complex, like the hexaploid level (corresponding to 42 chromosomes), known only from *V. coreana* Briq. [66] and *V. transjenisensis* Kreyer [67]. The results compiled in the present study allow us to conclude that the complexity within *V. officinalis*, in which polyploidization events are largely implicated, is much more than previously considered.

5. Concluding Remarks

At the family level, changes in basic chromosome number and genome size coincide with or precede major shifts in the evolutionary history of Valerianaceae. One interesting example is the arising of strong zygomorphic flower in *Centranthus* and *Fedia*, which is in both cases consecutive to a polyploidization event. Therefore, cytogenetic studies are essential for understanding the family, and, in this sense, we will follow our effort for providing new data of this type, especially in those genera poorly studied or unknown at different levels (i.e., genome size) such as *Plectritis*.

Acknowledgments

The authors acknowledge S Siljak-Yakovlev for useful comments for improving the chromosome count protocol, and J Suda and three anonymous reviewers for valuable suggestions on the draft. They thank M Bou Dagher-Kharrat, R Douzet, M Casanovas, C Hidalgo, Odile Hidalgo, JM Martin, N Martin, S Pyke, ML Revel-Hidalgo, K Romashchenko, À Romo and I Soriano for their assistance with collections, N Xena de Enrech and M-B Raymúndez for helpful comments, SC Brown and O Catrice for supplying *Petunia hybrida* and *Pisum sativum* used as internal standards, R Álvarez, J Comas, R López and R Martínez for technical support in flow cytometry, M Veny for keeping the collections of living plants, and S Pyke for the amelioration of the English language. The collaboration of the botanical gardens and herbaria listed in Table 1 is also acknowledged. This work was subsidized by the Dirección General de Enseñanza Superior, Spain (Project PB 97/1134), the Ministerio de Ciencia y Tecnología, Spain (projects CGL2007-64839-C02-01 and C02-02/BOS) and the Generalitat de Catalunya ("Ajuts a grups de recerca consolidats" 2005/SGR/00344). O Hidalgo received an MICINN postdoctoral grant from the Ministerio de Ciencia e Innovación, Spain and S Garcia a JAE Doc contract from the CSIC, Spain.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 480873, 8 pages
doi:10.1155/2010/480873

Research Article

Genome Size Variation in *Malus* Species

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Received 22 January 2010; Revised 12 March 2010; Accepted 12 March 2010

Academic Editor: Johann Greilhuber

The nuclear DNA content for 256 different accessions belonging to 26 primary *Malus* species and 20 species hybrids was estimated by flow cytometry using propidium iodide. Diploids ranged from 1.245 (*M. tschonoskii*) to 1.653 pg per 2C nucleus (*M. florentina*). As our study covered complete phylogenetic and geographic representation, preliminary conclusions between nuclear DNA content and geographical and taxonomic features could be drawn. The data indicated that species found far from the centre of origin in Asia clustered into separate sections and series and possessed higher DNA content. These are *M. trilobata* and *M. florentina* the only two species existing in South-East Europe on one hand; *M. ioensis* and 3x and 4x species *M.* ×*heterophylla*, *M.* ×*platycarpa, M. glaucescens, M. angustifolia, M. lancifolia* and *M. coronaria*—in East and Central North America on the other hand. A significantly decreased 1Cx DNA content was observed with the increase in ploidy for six species.

1. Introduction

Nuclear DNA amount and genome size are important biodiversity characters, whose study provides a strong unifying element in biology that has both practical and predictive uses [1]. At present, flow cytometry is the method most frequently used for determination of nuclear DNA content in plants. The data are summarized on the homepage of the Royal Botanic Gardens, Kew [2].

The apple, along with many of the important temperate fruit crops, belongs to the *Rosaceae* or rose family. The genus *Malus* Mill. comprises 25 to 47 species, depending upon the rank given to several taxa and the acceptance of putative hybrids. *Malus* classifications differ primarily in the taxonomic level at which infrageneric groupings of species are recognised. Rehder [3–5] proposed a classification system which is now accepted by later authors. Newer reports divided the genus *Malus* in six [6] or even in seven sections [7]. In China, the centre of origin of the genus *Malus,* about 80% of species are native and among them eight newly described species were recently recognized [8]. Most of the species intercross, and since self-incompatibility is common, seeds obtained from a botanic garden are mostly interspecific or intercultivar hybrids. Some taxa formerly listed as species are now classified as cultivated species because they are not known in the wild [6]. *Malus* is very diverse in morphology in nature, and the species represent a complicated system of ecotypes, forms, and varieties [9].

Most species are diploid ($x = 17$) and cross-pollinated, but Way et al. [10] list *Malus coronaria* from the American section Chloromeles as apomictic and either triploid or tetraploid. They also note deviations from the normal type in section Euromalus and Sorbomalus: *M. sikkimensis*, *M. hupehensis* and *M. toringoides* (apomictic and triploid), *M. sargenti* (apomictic and tetraploid), and *M. spectabilis* and *M. baccata* (non-apomictic and either diploid or tetraploid).

Arumuganathan and Earle [11] investigated the nuclear DNA content of more than 100 important plant species including *Malus*. Later, Dickson et al. [12] reported on the DNA content for 17 *Malus* species and eight cultivated apples, together with other genera of *Rosaceae*. Tatum et al. [13] determined the nuclear DNA content of various *Malus* species, hybrids, and cultivated apples included in the national core collection of *Malus* germplasm in the United States.

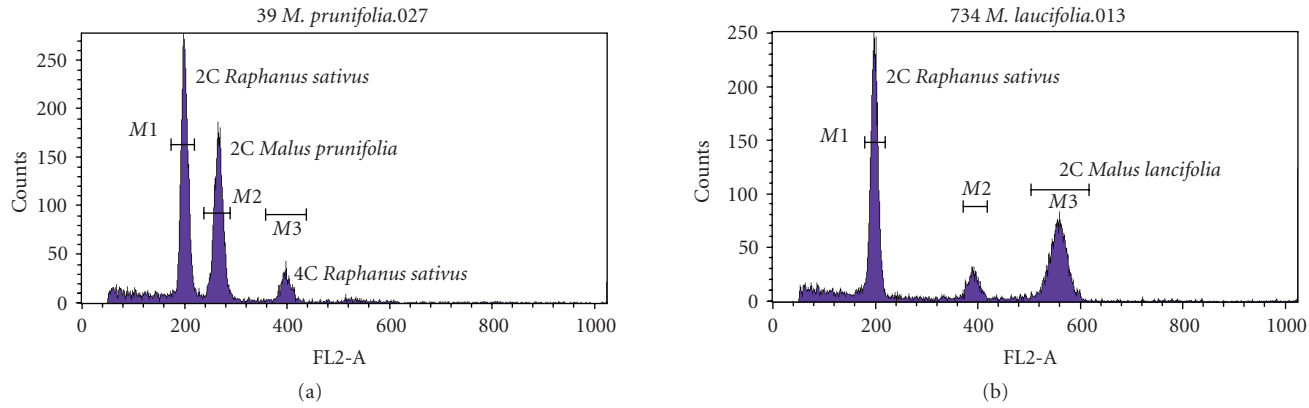

Figure 1: Flow cytometric DNA histograms of nuclei isolated from a diploid (*M. prunifolia)* and a tetraploid *(M. lancifolia*) *Malus* species with the internal standard *Raphanus sativus* (1.1 pg/2C).

The two main goals of this study were (1) to estimate the variation in the 2C nuclear DNA content for *Malus* species and of their species hybrids included in the *Malus* collection in the Institute for Breeding Research on Horticultural and Fruit Crops, Germany; (2) to inspect relationships between nuclear DNA content and geographical features of the taxa. Evaluation of this material representing all ploidy levels will provide an assessment of the level of interspecies variation for nuclear DNA content present in this collection and provides the most comprehensive overview of *Malus* taxa.

2. Materials and Methods

2.1. Experimental Material. Plant materials from 26 primary species and 20 species hybrids, altogether 256 accessions (different individuals) were collected from trees in the orchard of the *Malus* gene bank of the Institute for Breeding Research on Horticultural and Fruit Crops in Dresden, Saxony, Germany. Species concept and definition of higher rank taxa were based on the works of Rheder [5] and Langenfelds [14]. Seven diploid and four triploid *Malus* ×*domestica* cultivars were included. Young leaves were collected in May and June, stored on ice for the transport, and their nuclei were extracted immediately. The number of accessions investigated per species depended on the material available in the collection.

2.2. Nuclei Isolation and Staining. Nuclei were prepared for flow cytometric analysis using a Tris-$MgCl_2$ buffer according to the protocol of Galbraith et al. [15]. The extraction buffer consisted of per litre: 8.83 g tri-Natriumcitrat-Dihydrat, 9.14 g $MgCl_2 \times 6\ H_2O$, 4.19 g 3-(N-Morpholino) propanesulfonic acid (MOPS), and 5.0 g Triton x-100. Propidium iodide (PI) was added just before using the buffer (50 μg ml^{-1} final concentration). Preliminary experiments (not published) determined that the addition of RNAse was required (50 $\mu g\,ml^{-1}$ final concentration). Leaf tissues from apple (10 mg, stored on ice) and *Raphanus sativus* (2 mg) used as an internal standard were co-chopped with a new razor blade in 0.5 mL extraction buffer in a Petri dish. After dilution with additional 0.5 ml buffer and 1 minute staining time the homogenate was filtered through a 30 μm nylon mesh into a labelled test tube. During the preparation of 10 sample replications of each accession the samples were kept on ice. All 10 replications were measured immediately. The nuclear DNA content of radish is reported to be 1.1 pg/2C [16]. Seeds were sown in pots, and plants were grown in a greenhouse. Young healthy leaves of radish plantlets (3rd or 4th leaf pair) were used for measurements.

2.3. Flow Cytometry. Nuclei were analyzed using a laser Flow Cytometer FACScan (BECTON DICKINSON). PI was excited at 488 nm, and the emission was analysed at 590 nm. A minimum of 10.000 particles per sample were analysed. Mean fluorescence of the apple G1 peak was divided by the fluorescence reading of the G1 peak of the internal standard, multiplied by 1.1 pg/2C, and expressed in pg/2C nucleus.

2.4. Statistical Analysis. For each accession, 10 sample replications were prepared, measured, and analysed. Statistical analyses were performed using SigmaStat Version 3.10, Systat Software 2004. Because the majority of data were not normally distributed, differences in DNA content among the species were tested by nonparametric tests, either by the Mann-Whitney Test (in case of two samples) or by the Kruskal-Wallis Test together with Dunn's Multiple Range Test (in case of more than two samples) to determine significant differences.

3. Results

The nuclear DNA content of 256 accessions belonging to 26 primary species and 20 species hybrids of the *Malus* genus was estimated by flow cytometry using propidium iodide. Significant differences ($P < 0.05$) in 2C DNA content were detected within the *Malus* species analysing each ploidy level separately (Table 1). Figure 1 shows typical diploid and tetraploid histograms as analyzed by flow cytometry. The

Table 1: Nuclear DNA Content (pg/2C median; 25% and 75% value) of *Malus* species (N° of accessions used for measurements) with the results of the nonparametric Kruskal-Wallis Test together with Dunn's Multiple Range Test; medians with the same letter are not significantly different at 5% level. Each ploidy level was analysed separately. Classification was based on the works of Rehder [5] and Langenfelds [14].

Species	Section	Series	Nuclear DNA content (pg/nucleus)*				Results of the Kruskal-Wallis Test					
			N°	Median	25%	75%						
Ploidy level 2x = 34				1.484								
M. tschonoskii	Docyniopsis		1	1.245	1,150	1,283	a					
M. yunnanensis	Sorbomalus	Yunnanenses	2	1.337	1,320	1,350	a					
M. ombrophila	Sorbomalus	Yunnanenses	1	1.341	1,329	1,351	a	b				
M. kansuensis	Sorbomalus	Kansuenses	6	1.365	1,350	1,384	a	b				
M. honanensis	Sorbomalus	Yunnanenses	1	1.378	1,276	1,413	a	b				
M. prattii	Sorbomalus	Yunnanenses	1	1.397	1,386	1,406	a	b				
M. baccata	Euromalus	Baccatae	16	1.440	1,430	1,467		b				
M. ×zumi 2x	Euromalus	Baccatae	10	1.461	1,439	1,512		b	c			
M. ×dawsoniana	Sorbomalus	Kansuenses	1	1.464	1,455	1,478		b	c			
M. fusca	Sorbomalus	Kansuenses	5	1.464	1,460	1,470		b	c			
M. hupehensis 2x	Euromalus	Baccatae	2	1.466	1,451	1,475		b	c			
M. ×hartwigii	Euromalus	Baccatae	1	1.471	1,450	1,475		b	c			
M. ×adstringensis 2x	Euromalus	Baccatae	5	1.475	1,454	1,493		b	c			
M. ×moerlandsii	Euromalus	Pumilae	4	1.476	1,457	1,493		b	c			
M. ×purpurea	Euromalus	Pumilae	7	1.484	1,467	1,493			c	d		
M. sylvestris	Euromalus	Pumilae	11	1.484	1,460	1,509			c	d		
M. ×robusta	Euromalus	Baccatae	5	1.490	1,458	1,519			c	d		
M. ×atrosanguinea	Euromalus	Baccatae	1	1.493	1,449	1,493		b	c	d		
M. ×sublobata	Euromalus	Pumilae	4	1.497	1,459	1,550			c	d		
M. ×arnoldiana	Euromalus	Baccatae	3	1.499	1,470	1,529			c	d		
M. ×asiatica	Euromalus	Pumilae	4	1.500	1,470	1,514			c	d		
M. prunifolia	Euromalus	Pumilae	10	1.500	1,475	1,519			c	d		
M. sieversii	Euromalus	Pumilae	6	1.501	1,482	1,513			c	d		
M. orientalis	Euromalus	Pumilae	7	1.502	1,458	1,519			c	d		
M. spectabilis 2x	Euromalus	Pumilae	7	1.502	1,484	1,523			c	d	e	
M. ×scheideckeri	Sorbomalus	Sieboldiane	3	1.507	1,479	1,530			c	d	e	
M. floribunda	Sorbomalus	Sieboldiane	6	1.509	1,490	1,529			c	d	e	
M. ×domestica 2x	Euromalus	Pumilae	8	1.514	1,487	1,530			c	d	e	
M. ×micromalus	Euromalus	Baccatae	2	1.528	1,497	1,534			c	d	e	
M. sieboldii 2x	Sorbomalus	Sieboldiane	5	1.530	1,505	1,533				d	e	
M. transitoria	Sorbomalus	Kansuenses	5	1.535	1,514	1,551				d	e	
M. ×soulardii	Chloromeles		4	1.564	1,512	1,588				d	e	f
M. ioensis 2x	Chloromeles		4	1.565	1,523	1,667					e	f
M. trilobata	Eriolobus		3	1.638	1,621	1,667						f
M. florentina	Sorbomalus	Florentinae	3	1.653	1,635	1,660						f
Ploidy level 3x = 51				2.198								
M. hupehensis 3x	Euromalus	Baccatae	18	2.121	2,014	2,174	a					
M. sikkimensis 3x	Euromalus	Baccatae	7	2.165	2,148	2,180	a	b				
M. ×zumi 3x	Euromalus	Baccatae	1	2.194	2,143	2,229	a	b	c			
M. sieboldii 3x	Sorbomalus	Sieboldiane	3	2.193	2,183	2,201		b	c			
M. ×adstring 3x	Euromalus	Baccatae	3	2.201	2,191	2,211		b	c			
M. toringoides 3x	Sorbomalus	Kansuenses	7	2.202	2,172	2,254		b	c			
M. spectabilis 3x	Euromalus	Pumilae	1	2.206	2,195	2,216		b	c			
M. halliana	Euromalus	Baccatae	3	2.209	2,174	2,217		b	c			
M. komarovii 3x	Sorbomalus	Kansuenses	5	2.232	2,210	2,261			c	d		

Table 1: Continued.

Species	Section	Series	Nuclear DNA content (pg/nucleus)*				Results of the Kruskal-Wallis Test				
			N°	Median	25%	75%					
M. ×*domestica* 3x	Euromalus	Pumilae	6	2.270	2,252	2,296			c	d	
M. sargentii 3x	Sobomalus	Sieboldiane	2	2.285	2,197	2,307				d	
M. ioensis 3x	Chloromeles		2	2.414	2,396	2,426				d	
M. coronaria 3x	Chloromeles		3	2.428	2,183	2,462				d	
Ploidy level 4x = 68				3.007							
M. sikkimensis 4x	Euromalus	Baccatae	2	2.851	2,815	2,894	a				
M. sieboldii 4x	Sorbomalus	Sieboldiane	5	2.879	2,832	2,938	a				
M. komarovii 4x	Sorbomalus	Kansuenses	2	2.917	2,847	3,013	a	b			
M. sargentii 4x	Sorbomalus	Sieboldiane	4	2.918	2,891	2,958	a	b			
M. toringoides 4x	Sorbomalus	Kansuenses	1	2.958	2,947	2,979	a	b	c		
M. lancifolia	Chloromeles		1	3.007	2,969	3,035			c	d	
M. glaucescens	Chloromeles		1	3.102	3,082	3,197				d	e
M. angustifolia	Chloromeles		1	3.116	3,101	3,122			c	d	e
M. ×*platycarpa*	Chloromeles		4	3.151	3,118	3,177				d	e
M. coronaria 4x	Chloromeles		6	3.186	3,115	3,221					e
M. ×*heterophylla*	Chloromeles		1	3.193	3,167	3,216					e
Ploidy level 5x-6x											
M. sikkimensis 5x	Euromalus	Baccatae	1	3.540							
M. sargentii 5x	Sorbomalus	Sieboldiane	1	3.874							
M. sargentii 5x-6x	Sorbomalus	Sieboldiane	1	4.286							

* Estimation based on propidium iodide staining by using the internal standard radish (1.1 pg/2C).

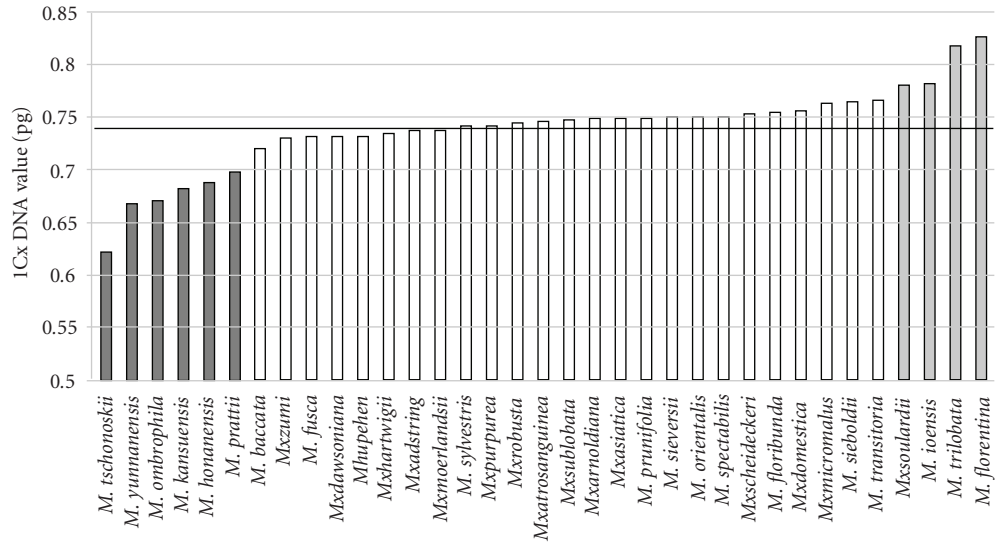

Figure 2: 1Cx DNA-values (medians) of the investigated diploid *Malus* species. Black line represents the median (0.742 pg) of all 34 investigated diploid *Malus* species. *Malus* species having 1Cx DNA-values more than 5% below the median are marked dark grey; species having 1Cx DNA-values higher than 5% above the median are marked light grey. The detailed statistical data are shown in Table 1.

nuclear DNA content over all ploidy levels (di-, tri-, tetraploids and higher 5x-6x) ranged from 1.245 to 4.286 pg per nucleus (Table 1).

Among the diploids, the difference between the species with the lowest (*Malus tschonoskii*—1.245 pg per nucleus) and the highest diploid value (*Malus florentina*—1.653) was 0.408 pg (= 1.328-fold) with a median DNA content of 1.484 pg per nucleus. The median of the cultivated apple (*M.* ×*domestica* 2x) determined by measurement of seven cultivars was 1.514 pg per nucleus.

Figure 2 demonstrates the 1Cx DNA values of the 34 investigated diploid *Malus* species. Six primary species (*M. tschonoskii; M. yunnanensis; M. ombrophila; M. kansuensis; M. honanensis; M. prattii*) were characterized by a value

Table 2: 1Cx-values (*Median) of *Malus* species demonstrating different ploidy levels. For statistical analysis, differences among the levels were tested by non-parametric tests: either by the Mann-Whitney Test (in case of 2 levels) or by the the Kruskal-Wallis Test together with Dunn's Multiple Test (in case of more than 2 levels). Species with the same letter are not significantly different at 5% level.

	$x = 2$	$x = 3$	$x = 4$	$x = 5$
M. ioensis	0.782a	0.805a		
M. coronaria		0.809a	0.796a	
M. sargentii		0.762a	0.730b	0.775a
M. sieboldii	0.765a	0.730b	0.720b	
M. ×domestica	0.757a	0.757a		
M. spectabilis	0.751a	0.735b		
M. komarovii		0.744a	0.729a	
M. toringoides		0.734a	0.739a	
M. ×adstringensis	0.738a	0.733b		
M. hupehensis	0.732a	0.707b		
M. ×zumi	0.731a	0.732a		
M. sikkimensis		0.722a	0.712b	0.708b

* Estimation based on propidium iodide staining by using the internal standard radish (1.1 pg/2C).

more than 5% below the 1Cx median (Figure 2—dark grey marked columns). *M. ionensis,* the only diploid species existing in East and Central North America, its species hybrids *M. ×soulardii*, *M. trilobata,* and *M. florentina*, the only two species existing in South-East Europe, with the highest nuclear DNA content of the diploid species were characterized by a value higher than 5% above the 1Cx median of the diploid species (Figure 2—light grey marked columns). 25 species including 17 species hybrid demonstrated 1Cx DNA contents in the interval from −5% to +5% of the 1 Cx median of the diploid species.

The nuclear DNA content of triploids varied between 2.121 pg and 2.428 pg per nucleus with a median of 2.198 pg. Triploids are documented in 13 species in the present collection. Only *M. halliana* exists as triploid accession alone. The tetraploids varied from 2.851 to 3.193 pg per nucleus with a median DNA content of 3.007 pg per nucleus. They were found in 11 species, five of them (*M. ×heterophylla, M. ×platycarpa, M. glaucescens, M. angustifolia,* and *M. lancifolia*) exist at the tetraploid level only. Three accessions were detected at the pentaploid and hexaploid levels.

Twelve species demonstrated accessions at different ploidy levels (Table 2). Three species contain accessions in three ploidy levels: *M. sargentii* and *M. sikkimensis* (3x, 4x, and 5x) and *Malus sieboldii* (2x, 3x, and 4x). According to the statistical analysis six of 12 species containing two or three ploidies demonstrated significant differences between the calculated 1Cx DNA contents (= monoploid genome size) of the different ploidy levels within these species (Table 2). For these six species an increase in ploidy results in a decrease in the 1Cx DNA content (*M. sargentii*; *M. sieboldii; M. spectabilis; M. ×adstringensis; M. hupehensis; M. sikkimensis*). The intraspecific variation of each species was expressed by the 25% and 75% values of the median calculated by the nonparametric Kruskal-Wallis One Way Analysis of Variance on Ranks (Table 1).

4. Discussion

Estimation of nuclear DNA content is one of the important applications of flow cytometry and a reliable and efficient method for characterization of plant nuclear DNA content. Nuclear DNA content represents an important biodiversity character with fundamental biological significance [17]. Studies on nuclear DNA content within *Malus* of the *Rosaceae* family were reported by Dickson et al. [12] and Tatum et al. [13]. The present study including 26 primary species and 20 species hybrids demonstrates the most comprehensive investigation of the genus *Malus.* The data presented provides a nearly complete geographic and phylogenetic representation (lacking four primary species).

Comparing the literature data two aspects have to be considered. First, in contrast to the above-mentioned literature based on one accession (individual) per species, the data presented here are the results of the investigation and analysis of more accessions of each species depending on the material available in the *Malus* collection of the Institute for Breeding Research on Horticultural and Fruit Crops. Second, Greilhuber et al. [18] considered the choice of a standard as critical for flow cytometric determination of DNA content and recommended that the standard used should have DNA values close to, but not overlapping, the 2C and 4C peaks of the target species. Price et al. [19] demonstrated that plant DNA data were more reliable when the internal standard was a plant, and nuclei from both the internal standard and the experimental plant were isolated and stained together to reduce any potential technical variation. For this reason the chosen internal standard for this experiment was radish with a nuclear DNA content of 1.1 pg [16]. Based on the optimized method for the present experiments in which histograms with coefficients of variation (CVs) of both standard and sample below 3 to 4% were obtained, the internal standard *Raphanus sativus* was used for the whole spectrum of ploidy

levels. For further experiments, the application of at least two internal standards bordering the diploid and the tetraploid levels of *Malus* is planned. Dickson et al. [12] used for the *Malus* experiments chicken erythrocytes nuclei that were previously fixed and stained, with a nuclear DNA content of 2.33 pg DNA per nucleus. Tatum et al. [13] co-chopped experimental apple leaves together with maize (5.35 pg DNA per nucleus). The present data differed in average by 2% from the corresponding species analysed by Dickson et al. [12]. A bigger difference exists to the data of Tatum et al. [13] whereas variations in both directions occurred. Tatum et al. [13] have already mentioned their differences to the former data of Dickson et al. [12]. The variation in nuclear DNA content between the lowest (*M. tschonoskii*) and the highest diploids (*M. trilobata*) was 0.408 pg corresponding to the data of Dickson et al. [12] investigating the same species and represents 27.5% of the median DNA content for diploid species. This variation within a ploidy level for the genus *Malus* is rather small when compared to other genera. Comparable studies investigating species within a genus demonstrated 1.7-fold difference of 2C DNA content in 34 Central European diploid species of *Cirsium* [20], while fourfold difference in DNA content was reported among 25 diploid species of *Lactuca* [21] and 23 *Trifolium* species [22]. Our investigations in *Pyrus* using 17 different species demonstrated only a difference of 0.067 pg (data not published). For analyzing intraspecific variation of each species (Table 1) more detailed investigations using adequate numbers of accession per species are necessary. Bennett et al. [23] characterized anthocyanins as a potential cause of significant error, inhibitor for propidium iodide DNA staining, and finally for genome size estimations. Elaborating the method of flow cytometric analysis in *Malus* special attention was given to the release of phenolics known as of ubiquitous distribution in *Rosaceae.* Based on the described optimized method for the apple material no changes of the DNA fluorescence were observed measuring the same sample in different time intervals in contrast to investigations done with *Pyrus* (data not published).

Previously, Schuster and Büttner [24] investigated chromosome numbers of many accessions (individuals) from the same *Malus* wild species collection in Dresden and reported different ploidy levels within eight species. They suggested that differences in ploidy in the same species can be attributed to frequent hybridization within the genus *Malus.* This hypothesis is supported by the large number of species hybrids available compared to the primary species (Figure 2). According to Ellstrand et al. [25], hybridization occurs most frequently in those genera characterized by a perennial habit, out crossing breeding system, and reproductive modes that are able to stabilize genetic hybridity. The present data of nuclear DNA content confirmed the results of chromosome counting by Schuster and Büttner [24]. By increasing the number of investigated accessions different ploidy levels could be determined in four additional *Malus* species. Comparing the literature data of *Malus* expressing different ploidy levels within species [10, 12, 13] *Malus komoravii* was described as tri- and tetraploid for the first time. The seeds for these accessions originated from in situ locations.

For a detailed analysis between nuclear DNA contents of accessions with different ploidy levels within the same species, the 1Cx DNA content was calculated of all accessions per ploidy and species (Table 2). 12 species demonstrated different ploidies. According to the statistical analysis (differences among the levels tested by non-parametric tests) the 1Cx DNA content becomes significantly lower with increasing ploidy level in six species (*M. sargentii*; *M. sieboldii; M. spectabilis; M. ×adstringensis; M. hupehensis; M. sikkimensis*). Leitch and Bennett [26] summarized a large-scale study of genera and families and concluded that (i) mean 1C DNA amount did not increase in direct proportion with ploidy, and (ii) mean DNA amount per basic genome tended to decrease with increasing ploidy. It was suggested that the loss of DNA following polyploidy formation, or genome downsizing, may be a widespread phenomenon of considerable biological significance. Molecular investigations of plant nuclear DNA content have shown that most genome size variability is associated with differences in repetitive DNA content, and in all plants investigated, the most significant contributions to genome size are due to a class of mobile DNA called retroelements or retrotransposons [27]. In the present results four primary species and two species hybrids (including *Malus ×domestica*) exhibit no genome downsizing in polyploids.

Bennett [28] suggested that interspecific variation in DNA content has adaptive significance, and it is correlated with the environment and geographical distribution. *Malus* species originated from several parts of the world and are geographically distributed from the 1st to the 5th climatic zone [29]. According to Tatum et al. [13], triploids and higher polyploids *Malus* are found mainly in the colder (2nd and 3rd) climatic zones.

The present comprehensive investigation of 26 primary species and 20 species hybrids allows preliminary conclusions concerning the DNA content and the geographical distribution of *Malus* species and the taxonomical classification (Table 1 and Figure 2). The six primary species (*M. tschonoskii; M. yunnanensis; M. ombrophila; M. kansuensis; M. honanensis; M. prattii*) characterized by a value more than 5% below the 1Cx median (Figure 2) originated all from East and Central Asia, the centre of origin of the *Malus* genus and represent the section Docyniopsis (*M. tschonoskii)* and the series Yunnanenses of the section Sorbomalus. Four diploid species representing the highest nuclear DNA content were characterized by a value higher than 5% above the 1Cx median (Figure 2): *M. ionensis* and its species hybrid *M. ×soulardii.* These two species and all other species widespread in East and Central North America (*M. ×heterophylla*; *M. ×platycarpa*; *M. glaucescens*; *M. angustifolia*; *M. lancifolia*; *M. coronaria)* belong to the section Chloromeles. Species of this section always demonstrated the highest nuclear DNA content in the tri- and tetraploid level, suggesting a common origin (Table 1). *Malus trilobata* and *M. florentina*, the third and the fourth *Malus* species characterized by a value higher than 5% above the 1Cx median (Figure 2), represent the only two species existing in South-East Europe. *Malus trilobata* belongs as the only species to the Section Eriolobus, and *Malus florentina* is the

only species of the section Sorbomalus series Florentinae. Harris et al. [30] analysed nuclear rDNA and chloroplast DNA sequences and placed *Malus trilobata* and *Malus florentina* in the same cluster. The present measurement of the DNA content would support the results of Harris et al. [30]. Qian et al. [7] characterized *Malus florentina* as a new section Florentinae. 25 species including 17 species hybrids demonstrated 1Cx DNA contents in the interval from −5% to +5% of the median of the diploid species. All species belong to the Section Euromalus and Sorbomalus, series Sieboldii and Kansusensis, and have native distribution in Asia and Europe with the exception of the species *M. fusca* spread on the West coast of North America.

On the basis of the present study, it is apparent that species growing far from the centre of origin cluster in separate sections and series and possessed higher DNA content. These are *Malus trilobata* and *Malus florentina* the only two species existing in South-East Europe on one hand; *M. ioensis* and 3x and 4x species *M.* ×*heterophylla*, *M.* ×*platycarpa*, *M. glaucescens, M. Angustifolia, M. Lancifol*ia, and *M. coronaria* in East and Central America. Bures et al. [20] found a negative correlation in *Cirsium* with eastern limits of distribution. Further studies should include the four missing species of the section Sorbomalus, series Kansuenses (*M. xiaojinesis),* and the section Docyniosis (*M. doumeri*, *M. melliana,* and *M. laosensis)* to provide a complete analysis of the genus.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 758260, 7 pages
doi:10.1155/2010/758260

Research Article

Genome Sizes in *Hepatica* Mill: (Ranunculaceae) Show a Loss of DNA, Not a Gain, in Polyploids

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Received 8 December 2009; Accepted 25 February 2010

Academic Editor: Johann Greilhuber

Genome size (*C*-value) was applied anew to investigate the relationships within the genus *Hepatica* (Ranunculaceae). More than 50 samples representing all species (except *H. falconeri*), from wild and cultivated material, were investigated. Species of *Hepatica* turn out to be diploid ($2n = 14$), tetraploid ($2n = 28$), and a possible pentaploid. The somatic nuclear DNA contents (2C-value), as measured by flow cytometry with propidium iodide, were shown to range from 33 to 80 pg. The Asiatic and American species, often considered subspecies of *H. nobilis*, could be clearly distinguished from European *H. nobilis*. DNA content confirmed the close relationships in the Asiatic species, and these are here considered as subspecies of *H. asiatica*. Parents for the allotetraploid species could be suggested based on their nuclear DNA content. Contrary to the increase in genome size suggested earlier for *Hepatica*, a significant (6%–14%) loss of nuclear DNA in the natural allopolyploids was found.

1. Introduction

Phylogenies based on restriction sites of chloroplast and ribosomal DNA and morphological and cytological variations indicate that the genus *Hepatica* Mill. should be subsumed within genus *Anemone* (L.) [1]. However there are numerous gaps in the available molecular data [2].Therefore it seems better to refrain from creating new generic or infrageneric taxa in *Anemone* and keep the classical concept of *Hepatica* [3]. *Hepatica falconeri* (Thomson) Steward is sometimes placed in Anemone and sometimes in Hepatica [1, 2]. However, three of the total eight Hepatica taxa are supposed to be allotetraploids with *H. falconeri* as one of the parents [4]. Therefore it is here attributed to *Hepatica.* The genus comprises about 12 species disjunctly distributed in the temperate zones of Europe, North America, and East Asia. They can be arranged in two sections: the mainly diploid section *Hepatica* with $2n = 14$ and the mainly polyploid section *Angulosa* (Ulbr.) Nakai with $2n = 28$. They are popular as spring-flowering rock garden plants. Almost all species are in cultivation, and enthusiasts especially in Japan grow many cultivars. The boundaries between taxa of various ranks are still a subject of dispute. Weiss-Schneeweiss et al. [4] found incongruent topologies based on nuclear ITS and chloroplast *mat*K sequences. Commichau [5] arranged the American and Asiatic species (except *H. maxima,* Nakai) as varieties under *H. nobilis* Schreb. Dezhi and Robison [6] regarded *H. yamatutae* Nakai as synonymous with *H. henryi* (Oliv.) Steward and *H. asiatica* Nakai to be a variety of *H. nobilis* Schreb. So there is ample scope for further investigation. Although recent studies [7] have substantially clarified systematic relationships within *Hepatica*, some new problems did arise. The classical taxonomic traits based on morphology and geographical proximity are here supplemented with data on nuclear DNA content. More than 50 different accessions representing all known species except *H. falconeri* (Thomson) Steward, and commonly available hybrids, were measured in an attempt to better understand the infrageneric relationships and to gain insight into the origin of some of the cultivars.

Nuclear DNA content which can conveniently be measured by flow cytometry using propidium iodide (PI), a stoichiometric DNA stain that intercalates in the double helix, is more and more exploited for taxonomic purposes. Where many species in a genus have the same chromosome numbers, differences in nuclear DNA content, when present,

have proven to be very effective in delimiting infrageneric divisions in a number of taxa [7–18]. Flow cytometry can therefore be considered as a quick and useful method for understanding systematic relationships. Genome sizes are evaluated here in combination with available morphological, geographical, and molecular data. Therefore the here proposed taxonomy is not a single character taxonomy based on genome size alone. Moreover, Greilhuber [19] has clearly shown that intraspecific variation of genome size is much less than assumed. Mabuchi et al. [7] concluded, based on their determination of the nuclear DNA content, that there was an increase in DNA content after tetraploidisation, a not very frequent occurrence [6, 20, 21]. In this study, nuclear DNA content was used anew to test the hypothesized origins of the polyploid taxa and to infer the relationships among the Asian endemic species.

The evolution of genome size (*C*-value) [17] has received increased attention during recent years. Primitive angiosperms are now supposed to have had small genomes; increases up to a factor 1000 have occurred independently in various modern taxa [22]. Flow cytometry was successfully used to measure the 2*C*-value for several genera [7, 9–18]. In this paper it is shown, using several accessions for most species, that intraspecific variation is low in *Hepatica* species. This enabled us to differentiate between intraspecific variation and hybrids and to indicate the parentage of some cultivars.

2. Materials and Methods

2.1. Plant Material. Plant material was obtained from the collections of J. Peters, Germany; G. Dunlop (N. Ireland); M. Myers, UK; J. Massey and E. Myrholt**,** Norway. Where possible, material of known wild origin was used, and care was taken to ensure correct identification of all the material. Vouchers of the species are in the Herbarium of Leiden (L).

2.2. Flow Cytometric Measurement of Nuclear DNA Content. For the isolation of nuclei, about 0.5 cm^2 of adult leaf tissue was chopped together with a piece of *Agave americana* L. "Aureomarginata" as an internal standard. The chopping was done with a new razor blade in a Petri dish in 0.25 ml nuclei-isolation buffer to which 0.25 mg RNase/ml was added [8]. After adding 1.5 ml propidium iodide (PI) solution (50 mg PI/l in isolation buffer) the suspension with nuclei was filtered through a 30 μm nylon filter. The fluorescence of the nuclei for each sample was measured half an hour and one hour after addition of PI, using a Partec CA-II flow cytometer. The optical path contained a HBO mercury lamp, filters KG1, and BG12, dichroic mirror TK500, filter OG570, and a Leitz 50 $\times$ 1 water immersion objective. Data were analyzed by means of DPAC software (Partec GmbH). The 2C DNA content of the sample was calculated as the sample peak mean, divided by the *Agave* peak mean, and multiplied with the amount of DNA of the *Agave* standard. At least three different nuclear isolations were measured twice (= after 30 and 60 minutes) with at least 5000 nuclei for each measurement. Therefore the average data for each accession presented here are based on at least 6 DNA measurements. Most histograms revealed a Coefficient of Variation (CV) of less than 5%. The standard deviation was calculated for the DNA content of each species, using all relevant measurements. *Agave americana* "Aureomarginata" was chosen as internal standard, because it has a convenient amount of DNA relative to *Hepatica*. Moreover, it is available year-round, it does not mind several weeks without water, and being a large plant, it can serve numerous determinations, thereby further reducing variation in readings. It also has a low background in PI measurements and shows a single G_0 peak, almost lacking G_2 arrest. Fresh male human leucocytes (2C = 7.0 pg; 1 pg(= 0.978×10^9 base pairs) [23]) were chosen as primary standard [24]. This yields 2C = 15.9 pg for nuclei of *Agave americana*.

3. Results

All known *Hepatica* species (except *H. falconeri*) were investigated experimentally by flow cytometry. Species are listed with increasing nuclear DNA content (Table 1). A low intraspecific variation is found in most cases. The interspecific variation shows that genome size in diploid *Hepatica* varies between 33.0 and 36.3 pg (Tables 1 and 3). The difference between the highest and lowest DNA contents is about 3 pg, equivalent to nearly 3×10^9 base pairs. The tetraploids vary from 53 to 70 pg and one of the cultivars is likely pentaploid with 79.9 pg.

3.1. Section Hepatica (Table 1). (*H. americana* (DC.) Ker Gawl., *H. americana* ssp. *acuta* (Pursh) Zonn., *H. asiatica* Nakai ssp. *asiatica*, *H. asiatica* ssp. *japonica* (Nakai) Zonn., *H. asiatica* ssp. *insularis* (Nakai) Zonn., *H. asiatica* ssp. *pubescens* (Hiroe) Zonn., *H. maxima* (Nakai) Nakai, and *H. nobilis* Schreb.)

Fourteen accessions were measured for *H. nobilis*. They originated from Western and Eastern Europe, from Sweden to Spain. The DNA *C*-values have a rather narrow range of variation. They clearly differ from the American and Asiatic taxa. The value for *H. nobilis* is of average 33.0 pg whereas those for *H. americana* and the Asiatic species are 34.8 and 34.8–36.3 pg, respectively. Two taxa are distinguished for North America: *H. americana* (DC.) Ker Gawl. and *H. acutiloba* DC. (or *H. nobilis* var. *acuta* (Pursh) Steyerm). *Hepatica americana* grows on acid soil and has rounded leaflets whereas *H. acutiloba* has pointed leaflets and grows on limestone. However, they don't differ in nuclear DNA content (Table 1). Moreover both rounded and acute leaflets are found within *H. nobilis* and *H. japonica.* The former taxa are here considered as subspecies: *H. americana* ssp. *americana and H. americana* ssp. *acuta.*

Hepatica asiatica ssp. *japonica* is often considered as a variety or subspecies of *H. nobilis.* However, its genome size is more in line with the other Asiatic species. The Japanese *H. asiatica* ssp. *pubescens* is confirmed to be tetraploid. Its genome size is in line with it being an autotetraploid of *H. asiatica ssp. japonica*. *H. maxima* is restricted to the Ullung

Table 1: Hepatica species with their nuclear DNA content in pg, average for the species, standard deviation, and origin.

Hepatica species	2C DNA in pg	Average	Standard deviation	Origin (as = received as)
Section Hepatica				
H. nobilis Schreber	32.7	**33.0**	**0.9**	Sweden
H. nobilis Schreber	32.7			Poland
H. nobilis Schreber	33.2			hort. blue flower
H. nobilis Schreber	32.3			hort. pink flower
H. nobilis Schreber	32.9			hort. white flower
H. nobilis Schreber	32.7			hort. Rubra plena
H. nobilis Schreber	33.7			Sweden, forma Plena
H. nobilis Schreber	33.3			"Crenatiloba"
H. nobilis Schreber	33.9			Marmor Crenata'
H. nobilis Schreber	33.3			Walter Otto' (double)
H. nobilis Schreber	33.2			as var glabrata, Sweden
H. nobilis Schreber	33.2			as var glabrata, S.Oeland
H. nobilis Schreber	32.3			as var pyrenaica
H. americana ssp. *acuta* (Pursh) Zonn.	34.6	**34.8**	**1.0**	USA, on limestone
H. americana ssp. *americana* (DC.) Ker Gawl.	34.9			USA, on acid soil
H. maxima (Nakai) Nakai	35.9	**34.8**	**2.2**	Korea, BSWJ 4344
H. maxima (Nakai) Nakai	34.0			Korea
H. maxima (Nakai) Nakai	34.4			Korea, Ullung Do
H. asiatica Nakai ssp. a*siatica*	36.6	**36.3**	**1.4**	China
H. asiatica Nakai ssp. a*siatica*	35.9			Korea
H. asiatica ssp. *insularis* (Nakai) Zonn.	34.7	**35.4**	**1.5**	Korea, BSWJ 859
H. asiatica ssp. *insularis* (Nakai) Zonn.	36.1			Korea, Cheju Do
H. asiatica ssp. *japonica* (Nakai) Zonn.	35.6	**35.8**	**0.6**	Japan, blue flower
H. asiatica ssp. *japonica* (Nakai) Zonn.	36.4			Japan, pink flower
H. asiatica ssp. *japonica* (Nakai) Zonn.	35.4			Japan
H. asiatica ssp. *pubescens* (Hiroe) Zonn.	70.0	**70.0**	**2.9**	Japan
H. asiatica ssp. *pubescens* (Hiroe) Zonn.	79.9	**79.9**	**0.4**	Japan, "Tenjinbai"
Section Angulosa (Ulbr.) Nakai				
H. falconeri (Thomson) Steward	25.9*			Kashmir, Pakistan
H. henryi Steward	53.2	**53.0**	**2.9**	China
H. henryi Steward	52.7			via E. Myrholt
H. transsilvanica Fuss	55.5	**54.2**	**2.5**	Lilacina'
H. transsilvanica Fuss	54.4			Bulgaria
H. transsilvanica Fuss	54.2			Elison Spence'
H. transsilvanica Fuss	54.2			Loddon Blue'
H. transsilvanica Fuss	52.9			
H. yamatutae Nakai	58.2	**58.3**	**2.1**	China, Emei Shan
H. yamatutae Nakai	59.5			China, "Marmorata"
H. yamatutae Nakai	58.9			China, black leaf
H. yamatutae Nakai	56.4			China, Emei Shan

* Recalculated from [7]

island off the Korean coast. It grows in a humid, largely frost-free environment. So it comes as no surprise that it is by far the largest plant in leaf and flower of this genus. It is also set apart in ITS and *mat*K trees and isozyme profile from the other Asiatic species. So it might have diverged stronger, but this is not reflected in its similar genome size. The diploid Asiatic species that have a similar genome size are often considered as varieties of *H. nobilis.* They are closely related and are here transferred to subspecies status under *H. asiatica* as ssp. *asiatica*, ssp. *insularis*, ssp. *japonica,* and ssp. *pubescens*.

3.2. Section Angulosa (Ulbr.) Nakai (Table 1). (*H. falconeri* (Thomson) Steward, *H. henryi* (Oliv.) Steward, *H. transilvatica* Fuss, and *H. yamatutai* Nakai)

Hepatica falconeri has by far the lowest amount of DNA of any *Hepatica* species. If its genome size is recalculated from Mabuchi et al. [7] with *Hordeum vulgare* "Sultan" = 10 pg instead of 11.12 (see discussion) and brought in line with other results here presented, it has 25.9 pg. This might indicate an early split-off from the genus, but there is no further evidence to substantiate this. The diploid *H. falconeri* seems to be one of the parents of the three allotetraploids. This is based on the strongly crenate leaves of both *H. falconeri* and the tetraploids, their geographical proximity and the additive DNA *C*-values from *H. asiatica* and *H. falconeri*.

H. henryi with 53.0 pg has been attributed to the parents *H. falconeri* × *H. asiatica* [4]. This would result in a plant with (25.9 + 36.3) = 62.2 pg). This value is higher than the value found here indicating a loss of 14.8% of DNA in this allotetraploid. A similar loss is found for *H. transsilvanica* with 54.2 pg suggested to be an allotetraploid of *H. falconeri* × *H. nobilis.* That would result in a plant with (25.9 + 33.0) = 58.9 pg, an 8% loss. *H. yamatutae* comes from the same areas as *H. henryi* but has 58.3 pg nuclear DNA, 5.3 pg more, supporting the conclusion that these taxa are distinct and worthy of recognition. *H. yamatutae* with 58.3 pg comes closer to a possible cross of *H. falconeri* × *H. asiatica* (61.7 pg). Its low loss of nuclear DNA suggests a more recent origin. However *H. yamatutae* and *H. henryi* might have undergone different genome reshapings after polyploid forming, depending on a combination of genetic and ecological factors.

3.3. Comparing Natural and Artificial Hybrids (Table 2). Nuclear DNA value in *Hepatica* can also be of use in determining the origin of artificial hybrids. Many of the numerous *Hepatica* cultivars are of hybrid origin and the parentage is known in most cases. For this study 13 cultivars of garden origin were investigated. When the putative hybridisation is between species with distinct DNA *C*-values, the expected intermediate DNA value of putative hybrids is readily apparent.

The genome sizes for the diploid artificial hybrids *H. americana* ×*H. nobilis* and *H. maxima* × *H. nobilis* show a small loss of 3.5% compared to the average of their parents (Table 2). A low loss of 2.5% is also found for the tetraploid, artificial hybrids *H. transsilvanica* × *H. asiatica* ssp. *pubescens* and *H. transsilvanica* × *H. yamatutae*. Peculiar is the fact that for the triploid artificial hybrids *H. nobilis* × *H. transsilvanica* (*H.* × *media* Simonk.) and *H. japonica* × *H. transsilvanica* a gain of 3.2% is found.

Interesting is *H. asiatica* ssp. *pubescens* "Tenjinbai" with 79.9 pg whereas the tetraploid form from Japan *H. asiatica* ssp. *pubescens* has a genome size of only 70 pg.

4. Discussion

For two taxa, *H. americana* ssp. *acuta* (33.1 pg) and *H. americana* ssp. *americana* (33.2 pg), genome size was determined already by Feulgen densitometry more than 40 years ago [25]. These values are comparable with the present values of 34.9 and 34.6 pg. Nuclear DNA content for all species of *Hepatica* was determined recently by Mabuchi et al. [7]. However, they only present in their table (Table 3) the relative fluorescence compared with *Hordeum vulgare* with 11.12 pg. Doležel et al. [26] have shown, based on the combined results of four laboratories, that the 2*C*-value of *H. vulgare* is close to 10.0 pg of DNA. Moreover, in the Kew list of DNA *C*-values there are 17 values for Hordeum vulgare. The average value is 10.2 pg, close to the value of Doležel et al. It seems inappropriate that instead of this average value the second highest value with 11.12 pg was chosen as "prime value". The results of Mabuchi et al. [7] are recalculated with the value of 10.0 pg and expressed as a 2*C*-value in pg (Table 3). This resulted in still somewhat higher values for the diploids. Moreover, the DNA *C*-value for *H. falconeri* was adjusted further to bring it in line with the present values. Contrary to the similar results for the diploids, the two polyploids erroneously supposed to be derived from a doubling of *H. falconeri* are about 9% higher than expected [7]. This can be partly explained as done by Weiss-Schneeweiss et al., [4] by assuming other parents. Even then their values for the tetraploids seem (9%–15%) too high, compared with our results. This deviation in their values for the tetraploids is also demonstrated in their value of 89.2 for *H. nobilis* var. *pubescens* (M.Hiroe) Kitamura (here *H. asiatica* ssp. *pubescens*). This is 6.9% higher than the doubled value for their *H. nobilis* var. *japonica* Nakai (41.7 pg). They therefore suggest that the tetraploids have gained DNA compared with their putative diploid parents. This would imply a deviation from the general trend in angiosperms of genome size reduction after polyploidization [21, 27]. It cannot be excluded that the linearity of their apparatus was not appropriate [7]. Repetitive DNA elements, including retrotransposons, are major components of eukaryotic genomes and such elements have a tendency towards amplification [24]. Major decreases in genome size occur less frequently and such decreases have been observed following a doubling of the total genome by polyploidization [6, 20] or the change from perennial to annual habit [20]. The genome sizes presented here indicate (Table 1) that there actually is a slightly lower genome size of *H. asiatica* ssp. *pubescens* (70.0 pg) compared with the double value of its parent *H. asiatica* ssp.*japonica* (Nakai) Zonn.(35.8 pg). Natural allotetraploids seem to have lost

Table 2: Natural and artificial hybrids of *Hepatica* Mill. with their nuclear DNA content in pg, standard deviation calculated amount of nuclear DNA (see text), % DNA loss, ploidy, and cultivar name.

Hepatica hybrids	2C DNA in pg measured	St. dev.	2C DNA in pg Calculated from parents	% DNA loss or gain	Ploidy based on 2C-value	Cultivar name
H. americana ssp. *acuta* × *H. nobilis*	**32.5**	0.5	33.9	−3.5	2×	Schlyter'
H. maxima x H. nobilis	**32.5**	0.1	33.9	−3.5	2×	Frances'
H. nobilis × *transsilvanica* (× *media* Simonk.)	**43.7**	1.3	43.2	2.6	3×	Ballardii'
H. nobilis × *H. transsilvanica* (*H.* × *media* Si'monk.)	**44.1**		43.2		3×	Buis'
H. nobilis × *transsilvanica* (*H.* × *media* Simonk.)	**45.2**		43.2		3×	Marmorata'
H. americana × *H. transsilvanica*	**44.2**	1.9	44.4	−0.5	3×	Millstream Merlin'
H. asiatica ssp. *japonica* × *H. transsilvanica*	**47.1**	0.4	45.0	4.7	3×	Prof. F. Hildebrand'
H. transsilvanica × *H. pubescens* (as "Tenjinbai")	**59.9**	1.6	62.1	−3.1	4×	Röttgersbüttler Röschen'
H. transsilvanica × *H. pubescens* (as "Tenjinbai")	**59.0**		62.1		4×	Weinreichs Weisse'
H. transsilvanica × *H. pubescens*	**61.7**		62.1		4×	Prof. F. Hildebrand'
H. transsilvanica×*H. yamatutae*	**54.8**	2.0	56.2	−2.5	4×	Harvington Beauty'
H. transsilvanica×*H. yamatutae*	**55.1**	1.9	56.2	−2.0	4×	NT4'
H. transsilvanica Fuss	**54.2**	2.5	*58.9	−8.0	4×	data from Table 1
H. henryi (Oliv.) Steward	**53.0**	2.9	**62.2	−14.8	4×	data from Table 1
H. yamatutae Nakai	**58.3**	2.1	**62.2	−6.3	4×	data from Table 1
H. asiatica ssp. *pubescens*	**70.0**	2.9	71.6	−2.3	4×	data from Table 1
H. asiatica ssp. *pubescens* "*Tenjinbai*"	**79.9**	0.4	89.5	−10.7	5×	data from Table 1

* calculated with *H. falconeri* × *H. nobilis as parents.* ** calculated with *H. falconeri* × *H. asiatica as parents.*

8%–15% of their genome as it happens often with old tetraploids [21, 27], whereas the autotetraploid *H. asiatica* ssp. *pubescens* shows only a 2.3% loss. It has already been observed [4] that half of the four 5S RNA and 35S RNA sites is gradually lost in some populations of the tetraploids. It cannot be excluded that an extinct/not yet discovered relative of *H. falconeri* or *H. asiatica* with a lower amount of nuclear DNA was involved. Similar large decreases in genome size for tetraploids compared with their diploid parents were earlier reported for the tetraploid *Ranunculus acris* (13%) [18] and *Ranunculus ficaria* (24%) [28]. One wonders whether or not these losses of nuclear DNA (6.3%, 8%, and 14.8%) found here in the allotetraploids of *Hepatica* are indicative for the age of these tetraploids.

Interesting is *H. asiatica* ssp. *pubescens* "Tenjinbai" having with 79.9 pg the highest nuclear DNA content measured here for *Hepatica*. The tetraploid form from Japan *H. asiatica* ssp. *pubescens,* has a genome size of only 70 pg. If *H.* "Tenjinbai" is a pentaploid form of *H. asiatica* ssp. *pubescens* it would have lost 9% of its DNA and if it was a hexaploid form, it would have lost 24% of its DNA. The pentaploidy seems to be the most likely explanation, although hexaploidy cannot be excluded seeing the similar high DNA loss of 24% reported for the tetraploid *Ranunculus ficaria* [28]. Hiroe [29] reported on a hexaploid var. *pubescens* from Mountain Fujiwara. *Hepatica* "Tenjinbai" might be such a plant. However, Hara and Kurosawa [30] did find only tetraploid plants at this locality. It suggests that further molecular and cytological work is required to ascertain the true genetic make-up of this taxon. The artificial hybrids supposed to be between *H. transsilvanica* and *H. asiatica* ssp. *pubescens* "Tenjinbai" should result in plants with (79.9 + 54.2)/2 = 67 pg. However, a value of 60.2 pg is found for three different cultivars (Table 2), suggesting a DNA loss of 10%. More likely the second parent in this cross was just plain *H. asiatica* ssp. *pubescens* instead of *H.* "Tenjinbai" resulting in a calculated value of 62.1 pg for the hybrid and an actual DNA loss of 3.1%. This is in line with the loss in the other artificial tetraploid hybrids. The losses in the artificial tetraploids are thus lower than the losses in the natural tetraploids *H. henryi, H. transsilvanica,* and *H. yamatutae* where we find losses of nuclear DNA compared with their supposed parents of 8%, 14.8%, and 6.3%, respectively. This indicates that there is over time a slow but increasing loss of DNA in these natural allotetraploids that are of older age than the artificial hybrids mentioned above.

Weiss-Scheeweiss et al. [4] investigated the phylogenetic relationships of *Hepatica.* This was inferred from the maximum likelihood of the nuclear internal transcribed spacer ITS and the plastid *mat*K region, and also on karyotype morphology, banding patterns and rDNA localization. Nuclear and plastid sequences resulted in incongruent topologies mainly because of the position of some tetraploid taxa. Our results seem incongruent with both topologies obtained.

5. Evolutionary Considerations

Based on not only genome size but also leaf shapes, flower color, and geographical arguments, the following reasoning seems plausible. *Hepatica falconeri* is the basal species with only 25.9 pg of DNA [7] and could be a relict, surviving in Pakistan. There could have been a spread eastwards

Table 3: Genome sizes of Hepatica compared with results of Mabuchi et al. [7] and idem recalculated.

	This article	Mabuchi et al. [7] H. vulgare = 11.12 pg	recalculated H. vulgare = 10.0 pg	% difference between column B and D
H. falconeri (Thomson) Steward	25.9*	30.2	27.2	
H. nobilis Schreber	33.0	38.3	34.4	**4.2**
H. americana (DC.) Ker Gawler	34.6	40.3	36.2	**4.6**
H. americana ssp. *acuta* (Pursh) Zonn.	34.9	40.3	36.2	**3.7**
H. maxima (Nakai) Nakai	34.8	41.7	37.5	**7.8**
H. asiatica ssp. *insularis* (Nakai) Zonn.	35.4	41.7	37.5	**5.9**
H. asiatica Nakai ssp.*asiatica*	36.3	42.3	38	**4.7**
H. asiatica ssp. *japonica* (Nakai) Zonn.	35.8	41.7	37.5	**4.7**
H. henryi (Oliv.)Steward	53.0	65.8	59.2	**11.7**
H. transsilvanica Fuss	54.2	66.5	59.8	**10.3**
H. yamatutae Nakai	58.3	70.9	63.8	**9.4**
H. asiatica ssp. *pubescens* (Hiroe) Zonn.	70.0	89.2	80.2	**14.6**

* calculated from [7]

to Eastern Asia (*H. asiatica, H. maxima, H. asiatica* ssp. *insularis,* and *H. asiatica* ssp. *japonica*) westwards to Europe (*H. nobilis*), and via the bridges in the Miocene to eastern North America [31], in all cases with an increase in genome size. Apart from *H. asiatica* ssp. *pubescens* the other three tetraploids seem to be of allotetraploid origin with a crenate-leaved species like *H. falconeri* or of a related, unknown taxon as one parent. *Hepatica yamatutae* is endemic to the Emei Shan, Sichuan. This is inside the territory of the more widespread *H. henryi* (China: Hubei, Hunan, Shaanxi, Sichuan [7]). For *H. yamatutae* there is only a 6% loss of nuclear DNA, whereas a 12% loss was calculated for *H. henryi* from the same area. Maybe this and its small territory point to a more recent origin of *H. yamatutae* compared with *H. henryi* that is supposed to have the same parents.

6. Conclusions

Flow cytometry can be a useful tool to indicate the relationship and taxonomic status of *Hepatica* accessions. Although the DNA content is not unique to every taxon, many species (and some subspecies) can be identified using this method.

Taxa clearly different in nuclear DNA amount are considered good species. This does not mean that taxa with identical DNA amount, must always be considered as constituting a single species. The nuclear DNA amounts should always be evaluated in combination with morphological/molecular data, just as any other taxonomic characters. In some cases, the measured DNA value gives rise to questions about the perceived taxonomic relationship of certain taxa. The speed and cost effectiveness of measuring nuclear DNA content and its predicative accuracy makes it useful as a tool for identifying the origin of *Hepatica* taxa.

New Name Combinations

The above results have demonstrated that the Asiatic hepaticas are different in nuclear DNA content from *H. nobilis*. Therefore four diploid Asiatic taxa are arranged under *H. asiatica* as they are very similar in DNA 2*C*-value. They are also geographically connected and isolated from *H. nobilis*. The two American taxa, differing mainly in leaf shape and ecology, have similar genome sizes that differ from that of *H. nobilis*. They are better considered as subspecies from *H. americana*.

- *H. americana* ssp. *acuta* (Pursh) Zonn stat. nov. Basionym *H. triloba* Chaix var. *acuta* Pursh [32].
- *H. asiatica* ssp. *insularis* (Nakai) Zonn. stat. nov. Basionym: *H. insularis* Nakai [33].
- *H. asiatica* ssp. *japonica* (Nakai) Zonn. stat. nov. Basionym: *H. nobilis* var. *japonica* Nakai [34].
- *H. asiatica* ssp. *pubescens* (M.Hiroe) Zonn. Basionym: *Anemone hepatica* var. *pubescens* M. Hiroe [29] and Kadota [35].

Acknowledgments

Dr. J. F. Veldkamp (L) is thanked for critically examining the manuscript. The contribution of plants by the donors listed above is gratefully acknowledged.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 347254, 9 pages
doi:10.1155/2010/347254

Research Article

Icelandic Birch Polyploids—The Case of a Perfect Fit in Genome Size

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Received 31 January 2010; Accepted 22 April 2010

Academic Editor: Ilia Judith Leitch

Two birch species coexist in Iceland, dwarf birch *Betula nana* and tree birch *B. pubescens*. Both species are variable morphologically, which has been shown to be due to introgressive hybridization via interspecific hybrids. The aim of this study was to examine if the introgression could be related to genome size. We characterized 42 plants from Bifröst woodland morphologically and cytogenetically. The population consisted of diploid *B. nana* (38%), tetraploid *B. pubescens* (55%), and triploid hybrids (7%). Genome size was measured from 12 plants, using Feulgen DNA image densitometry (FDM) on spring leaf buds and flow cytometry (FCM) with dormant winter twigs. The use of winter twigs for FCM is novel. The average 1C-values for diploid, triploid, and tetraploid plants were 448, 666, and 882 Mbp, respectively. Monoploid genome sizes were found to be statistically constant among ploidy levels. This stability is in contrast to the different taxonomic positions of the di- and tetraploids and also contrasts with the frequent occurrence of genome downsizing in polyploids.

1. Introduction

Two species of *Betula* L. coexist in Iceland [1]: the diploid dwarf birch *B. nana* L. and the tetraploid tree birch *B. pubescens* Ehrh. *Betula nana*, a circumpolar species [2], grows up to one metre in height with procumbent, wide-spreading stems and a strong branching habit. *Betula pubescens* is a European species [3], represented by the subspecies *pubescens* Ehrh., which may grow up to 25 m tall with single or many stems (monocormic or polycormic type), and subspecies *tortuosa* (Ledeb.) Nyman is a shrub or low tree found in the mountain regions of northern Europe and is therefore called "mountain birch". *Betula pubescens* in Iceland has sometimes also been referred to as mountain birch due to its low stature and scrub-like growth form [4]. Elkington [5] compared birch from Iceland and Scotland and suggested that the morphological variability and the shrub-like appearance of Icelandic birch were due to genetic introgression from *B. nana* into *B. pubescens* via triploid interspecific hybrids. This was confirmed by both crossing experiments and population-based studies [1, 6]. Furthermore, these studies revealed that the introgression occurred in both directions, that is, not only from the diploid to the tetraploid species but also from the tetraploid to the diploid birch species, resulting in a high degree of variation in both *B. nana* and *B. pubescens*.

Due to this bidirectional introgression between the two *Betula* species, taxonomic delineation in Icelandic birch has been difficult, especially with the tree birch species *B. pubescens*. Morphological variation in birch plants from natural woodlands throughout Iceland was examined using both qualitative species-specific botanical characters and quantitative morphological variables [7]. The study revealed that Icelandic birch populations consisted mostly of introgressant types. Whereas about 11% of all plants examined were "morphologically pure" *B. nana*, not a single plant had all of the species-specific characters of *B. pubescens*. Indeed only 1% of the plants examined had more than 80% of the *B. pubescens* characters. But when the plants were sorted according to chromosome number, which was determined by direct chromosome counting of mitotic metaphases, three groups of birch emerged: diploid ($2n = 28$), triploid

Table 1: Plant materials, ploidy determination, morphology evaluation, and genome size measurements (C- and Cx-values in Mbp) from FDM and FCM.

Plant identity number	Ploidy by karyotype	DNA Ploidy	Morphology index	1Cx-values		2C-values	
				FDM-buds	FCM-twigs	FDM-buds	FCM-twigs
Bn-1	—	2x	—		453.4		906.8
Bn-2	—	2x	—		461.7		923.4
44	2x	2x	1	441.2		882.4	
46	2x	2x	3	431.8		863.6	
51	2x	2x	1	451.1		902.2	
72	3x	3x	4		443.4		1330.2
81	3x	3x	4	447.2	441.7	1341.6	1325.1
71	4x	4x	6	445.4	439.3	1781.4	1757.2
83	4x	4x	7	433.7	453.5	1734.8	1814.0
85	4x	4x	8		430.2		1720.8
92	4x	4x	10	439.7	438.4	1758.8	1753.6
95	4x	4x	9		448.2		1792.8

($2n = 42$), and tetraploid ($2n = 56$). The diploid ($2n = 28$) group consisted mainly of *B. nana* and *B. nana*-like plants, although some diploid plants showed intermediate "hybrid" morphology. The tetraploid group consisted of *B. pubescens*-like plants and a significant proportion of plants that had intermediate morphology. The triploid group (about 10% of all plants examined) showed no "typical" morphology, although there was a trend towards greater similarity to *B. nana*. The multivariate data analysis of morphological variables was able to separate diploid *B. nana* from tetraploid *B. pubescens* most of the time, but triploid hybrids were difficult to predict;-only half of them were assigned correctly.

The three genome groups in Icelandic birch were distinguishable statistically but their morphological variation overlapped in most of the distribution, implying bidirectional introgression [7, 8]. The question is whether this introgressive hybridization has affected genome size and if so, to what extent. There might be large-scale genomic recombination and shuffling following the hybridization and back-crossing, leading to genome size changes and significant variation within and between genome groups. Genome downsizing has been shown to be common in angiosperms [9], whereby genome size does not increase in direct proportion with ploidy. Genome size expansion due to transposition has also been documented, but there seems to be a large variation in the nature and extent of opposing forces to genome obesity [10, 11], making the direction of genome size change in a given species difficult to predict. On the other hand, there may be no deviation at all in genome size proportional to ploidy if there is sufficient (strong) selection pressure against genome-wide (neutral) introgression, especially in hybrid zones. For the hybrid zones to be stable, they require some form of divergent selection (intrinsic and/or extrinsic) to maintain differentiation (species integrity) despite extensive gene flow. Divergent selection has been suggested to contribute to heterogeneous genomic divergence and ecological speciation [12] and to play an important role in hybrid zone maintenance [13]. Our morphological [7, 8] and molecular [14] studies have supported the suggestion that Iceland is a birch hybrid zone, harbouring genetic variation which may be advantageous in subarctic regions.

The aim of the present study was therefore to get the first set of genome size data on Icelandic birch, including samples from all three ploidy groups (diploid, triploid, and tetraploid) from one of the most extensive birch woodlands in Iceland. Two approaches were used to measure genome size in these samples: (A) the Feulgen DNA image densitometry (FDM) method for squashed preparations of meristematic cells in newly bursting shoot buds and (B) the flow cytometry (FCM) method for cells from winter-dormant twigs. The genome size data generated in this study will undoubtedly contribute to the understanding of the process and mechanism of introgressive hybridization in Icelandic birch.

2. Materials and Methods

2.1. Plant Materials. Plant materials in this study came from the Bifröst woodland (64.76°N/21.59°W, 62 m.a.s.l., 11°C mean July temperature), by Lake Hredavatn in Borgarnes district, West Iceland. This is part of the largest continuous woodland area still in existence today. In this woodland, like most other woodlands in Iceland, both *B. nana* and *B. pubescens* grow most often side by side, although *B. nana* tends to be more dominant on wet ground, whereas *B. pubescens* occupies drier land. A total of 42 plants were selected within an area of about one km^2 and with a distance of at least 20 m between plants. The plants were marked in the field to enable a long-term study, whereby samples could be collected from the same plants in different visits over a number of years.

2.2. Morphological and Cytogenetic Characterization. For the morphological analysis, 30 leaves were collected from each plant. These were late leaves from long shoots, normally from the third to fifth positions from the shoot tip. Discrete, species-specific morphological characters were used to

determine the morphological variation of *B. nana* and *B. pubescens*. Eight characters including growth form, growth habit, and characteristics of leaf shape were examined qualitatively and scored as described previously [8], whereby each character was given two or three possible scores from zero (*B. nana*) to one or two (*B. pubescens*). Scores for leaf characters of each plant were assigned using average values from 30 leaves that had been pressed, sealed in plastic, and scanned. The scores of all characters were then combined for each plant into a single value, called a morphology index. This was assigned to place *B. nana* at the lowest ranks and *B. pubescens* at the highest.

The same sets of leaves were analysed quantitatively. The quantitative analysis of leaf characters was performed for nine variables as in [7], using the leaf morphology analysis program WinFolia (Regent Instruments, Quebec, Canada). The measured characters were analysed using the multivariate analysis of variance (MANOVA) and the linear discriminant analysis (LDA) described in [15].

Samples of bursting leaf buds in early spring (May-June) were collected in the field for chromosome analysis. The chromosome number of each plant was determined by direct chromosome counting, from metaphases prepared from shoot-tip meristems using the protoplast dropping method of Anamthawat-Jónsson [16]. The chromosome preparations were stained with the fluorescent dye DAPI (4, 6-diaminophenylindole) and visualized under a Nikon epifluorescence microscope (Eclipse 800) using 1000x magnification. The images were captured with a Nikon digital camera (DXM 1200F) using 12.5 million pixels. Chromosomes were counted from 10–20 metaphases from each plant.

2.3. Genome Size Determination Using Feulgen DNA Image Densitometry. Seven samples (individuals) of birch from the Bifröst woodland (Table 1) were collected in the field in May 2007 and were airmailed as twigs with early sprouting buds under a moist condition from Reykjavik to Vienna. Buds were detached and flooded with 4% phosphate-buffered formaldehyde (pH 7) in embryo cups and cut open. Bud meristems and leaf primordia were dissected out and fixed for 1.5 h at 20°C synchronously with embryonic roots and shoots from dry seeds of *Pisum sativum* "Kleine Rheinländerin" for internal standardization of the Feulgen reaction. Extensive washes with acetic-methanol (1 : 3) were performed, to remove formaldehyde from the tissue. The material was then rinsed extensively and finally stored in 96% ethanol at −20°C until use.

The Feulgen reaction was done as described in [17], that is, hydrolysis in 5N HCl for 90 min. at 20°C, then Schiff reagent for 90 min., with appropriate washes between all steps. Squashes were made in 45% acetic acid with birch and pea tissue particles placed side by side under separate cover slips on one slide. The slides were frozen over a cold plate and the cover slips were removed. The slides were air-dried and were kept dust- and light-protected for a maximum of a few days.

Measurement of DNA content was performed with video-based DNA image densitometry using the CIRES, version 3.1 (Kontron, Munich), using a 63x oil objective (Zeiss), green and neutral filters, the green channel of the video camera, and "local" background determination (around each nucleus separately). Three slides per plant were measured except on one occasion where two slides were examined, and usually 100 unreplicated nuclei per slide of birch and pea each were measured for DNA content determination. For each slide the 1C DNA content of birch was calculated using the birch/pea mean 2C DNA content ratio and 4.42 pg (1C) for pea [18]. The pg/Mbp conversion factor used is 1 pg = 978 Mbp [19]. Genome sizes are presented as 1C-values (holoploid genome size) and 1Cx-values (monoploid genome size), as proposed in [20, 21]. We use the term "DNA ploidy" [22] to indicate that ploidy level was inferred from DNA amounts instead of chromosome counts.

Figure 1: Winter twig of *Betula* from Bifröst woodland (plant no. 72)—decorticated twig as used for flow cytometry.

2.4. Genome Size Determination Using Flow Cytometry. In contrast to the Feulgen method using formaldehyde fixation, the FCM approach was shown to be unsuitable for sprouting leaf buds of birch because of an abundant amount of secondary metabolites. Icelandic birch, like the mountain birch of Scandinavia, is known to produce excessive amounts of primary and secondary metabolites in its leaves, especially the herbivory-related phenolic compounds [23]. We therefore looked for different materials for the FCM in this project.

Samples of young, winter-dormant twigs were collected from nine plants in the Bifröst woodland (Table 1) in November 2009. These plants were analysed morphologically and cytogenetically, except the two samples collected at this site from plants having the growth habit of *B. nana* (Bn-1 and Bn-2). Four of the karyotyped plants were analysed by FDM in 2007. The twig samples were sent by airmail to Vienna for the FCM study. The sample preparation for FCM began six days after the samples were collected in the field in Iceland.

About 1-2 cm long pieces of the youngest twig internodes were decorticated (Figure 1) and the cortex discarded, while the remaining twig was kept moist. Using a sharp razor blade, the decorticated twig segments were cochopped [24]

with *Solanum pseudocapsicum* leaves (1.29 pg DNA/1C [25] for internal standardization) in Otto's buffer I [26, 27]. The resulting nuclei isolate was filtered through a 30 μm mesh (Saatilene) and treated for 30 min. with RNase-A (0.15 mg/mL) at 37°C in a water bath. Thereafter, propidium iodide (PI, final concentration 50 μg/mL) dissolved in Otto's buffer II [26] was added to stain the nuclei at neutral pH for at least 1 h in a refrigerator. Decorticated winter twigs yielded enough nuclei for suitable histograms (Figure 2). Good separation of the 2C peaks of *Betula* and *Solanum* from debris is shown by the SSC versus fluorescence analysis in Figure 2 (insert). Tests for the interference of secondary compounds with nuclei fluorescence were made with chopped birch twigs and standard leaves alone and in combination. The measurements were made at constant instrument settings [27].

Genome size measurements were done using FloMax software on a CyFlow ML flow cytometer (Partec, Münster, Germany) equipped with a green laser (100 mW, 532 nm, Cobolt Samba, Cobolt AB, Stockholm, Sweden). The C-values were calculated for every run from the ratio of the mean fluorescence intensities of the standard and the birch G1 peaks. Usually the CV% of single peaks was lower than 3% and three runs (5000 particles/run) per preparation were sufficient. If the CV% was higher (up to maximal 5%), an additional one or two runs were done. Statistical tests (ANOVA and Wilcoxon pair test) were made using SPSS 10.

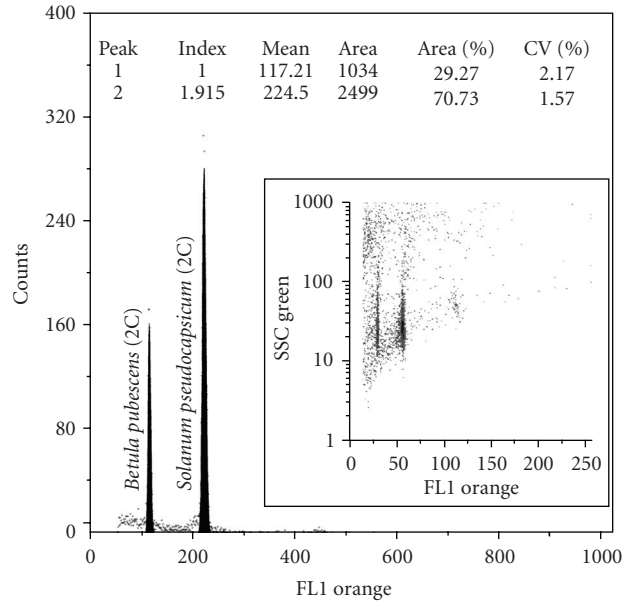

Figure 2: Flow histogram of *Betula pubescens* and the internal standard *Solanum pseudocapsicum*, with an inset showing scattergram of the side scatter (SSC green) and the fluorescence (FL1 orange) data of the particles measured.

3. Results

3.1. Ploidy and Morphological Characterization. The plants from Bifröst were characterized morphologically and cytogenetically (Figure 3) and were localized onto the quantitative introgression map of this woodland (Figure 4). Of the 42 plants examined, 16 plants (38%) were confirmed with accurate chromosome counts as being diploid ($2n$ = 2x = 28), 23 plants (55%) as tetraploid ($2n$ = 4x = 56), and 3 plants (7%) as triploid with 42 chromosomes (see examples in Figures 3(d)–3(f)). The percentages of different ploidy groups within the Bifröst population are similar to those found in all woodlands combined [7]. No aneuploid cells or aneuploid plants were detected. Ten of these plants were used in the genome size study (see Table 1): three diploid *Betula nana*, two triploid and five tetraploid *B. pubescens* plants.

The plants from Bifröst woodland represent the overall features of Icelandic birch in general. The plants are mostly morphologically introgressed birch although they are remarkably stable at the ploidy level, that is, the population consists only of diploid, triploid, and tetraploid plants, no aneuploids. But to what extent has Icelandic birch been modified, via introgressive hybridization? Botanically [28] the two *Betula* species differ mainly in that the dwarf birch species *B. nana* has orbicular or obovate-orbicular sessile leaves which are regularly crenate and are rounded at both leaf tip and base (similar to the leaves in Figure 3(a)), whereas the leaf shape of *B. pubescens* is usually cordate with dentate margins and a distinct petiole (similar to that of the tetraploid birch in Figure 3(c)). In the morphology index scale from zero for *B. nana* to 13 for *B. pubescens* (see Materials & Methods), given null introgression, the Icelandic diploid, triploid, and tetraploid groups from Bifröst were found to have average scores of 1.4 (higher than 0), 4.0 (relative intermediate), and 8.4 (much lower than 13), respectively. These are about the same average scores as those obtained from all major birch woodlands in Iceland combined [7]. The bidirectional introgression has clearly brought the two species closer to each other. Apart from these mean values, however, there is a considerable variation within ploidy groups. The diploid *B. nana* plants from Bifröst, for example, have a morphology index of 2 (Figure 3(a)), but 1 and 3 in the plants used in our genome size study (Table 1). The triploid plant in Figure 3(b) has a morphology index of 4, like the other two triploids examined in this study (Table 1). The tetraploid *B. pubescens* plant in Figure 3(c) has the typical index of 9 for this species in Icelandic woodlands, whereas those used in the genome size study show 6–10 in their morphology scale (Table 1).

The majority of the plants that were recorded as *B. nana* and *B. pubescens* in the field were found to be diploid and tetraploid, respectively. Triploid individuals, on the other hand, resembled either species as frequently as they appeared as morphological intermediates. But when quantitative species-specific characteristics of leaf shape were analysed, the plants from Bifröst woodland were separated into two groups only, that is, diploid and tetraploid groups (Figure 4). The triploids were not statistically distinguishable from *B. pubescens*. The same analyses applied to triploid plants from woodlands throughout Iceland: the triploids did not form an intermediate group [7].

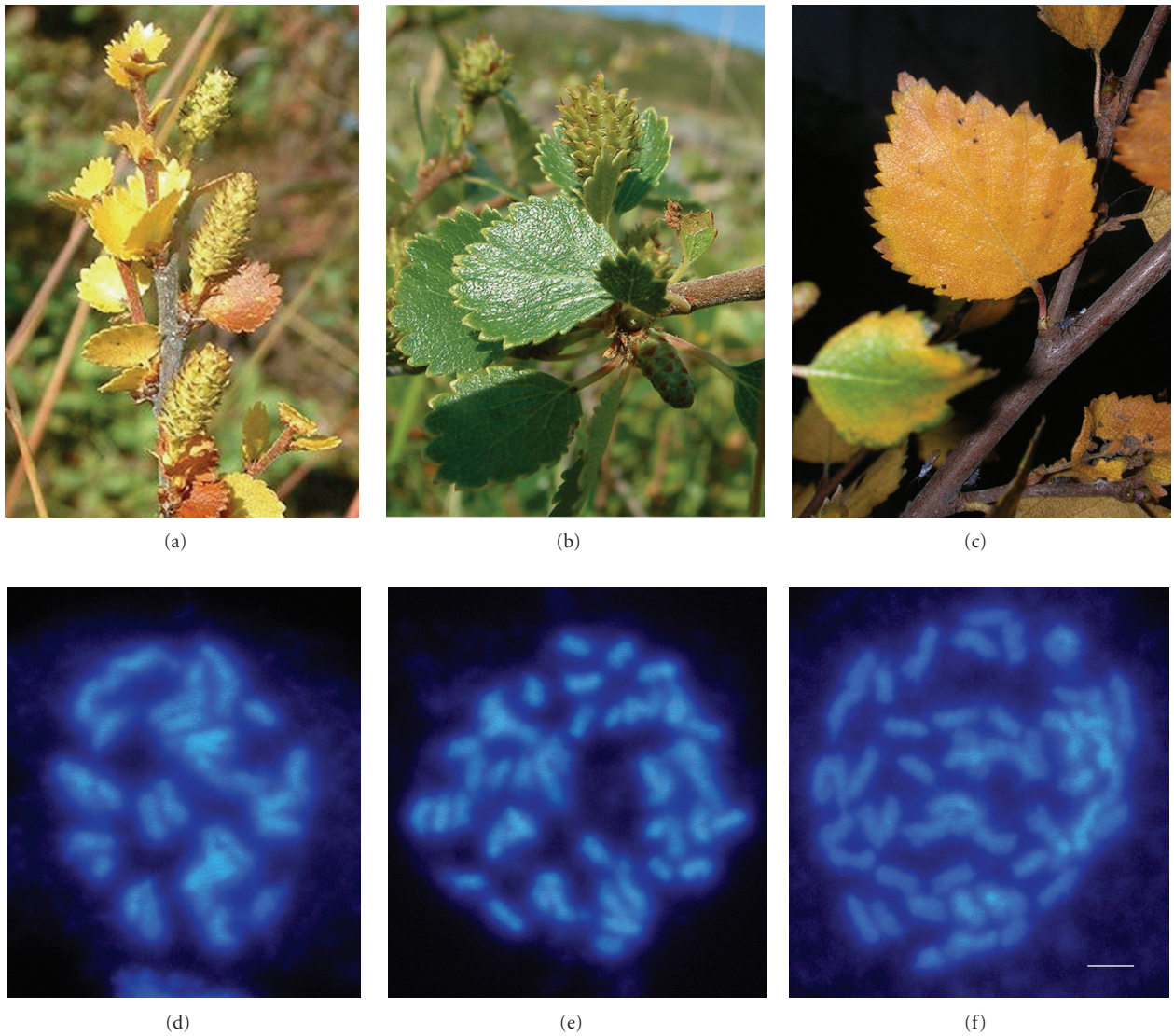

Figure 3: *Betula* from the woodland Bifröst, western Iceland—the plants and their chromosome numbers: (a) and (d) diploid *B. nana*, $2n = 2x = 28$, morphology index 2; (b) and (e) triploid interspecific hybrid, $2n = 3x = 42$, morphology index 4; and (c) and (f) tetraploid *B. pubescens*, $2n = 4x = 56$, morphology index 9. The scale bar represents 2 μm.

3.2. Genome Size in Icelandic Birch

3.2.1. Feulgen DNA Image Densitometry (FDM). Formaldehyde fixation and the Feulgen procedure resulted in delicately magenta-stained and often isolated birch nuclei, and a colourless transparent cytoplasm, in which solid bodies of polymerized condensed tannins were abundantly present in many but not all cells (compare with Greilhuber [29]). These tannin bodies were localized within the vacuoles. None or very little mitotic activity was observed in the slides. Mitotic nuclei, if found, conformed to the corresponding interphase 2C and 4C distributions. Somewhat higher CVs within some slides could be attributed to some background granulation. In the cells of the resting pea embryos used as standard, no tannins were found. Their nuclei were nonreplicating and conformed well to ploidy classes. Only 2C nuclei were used for calculation. Within-slide CVs in birch were mostly below 6%, with one exception of 7%, while in pea they were 1.5% to 3%.

Of seven birch samples (individual plants), three were "DNA diploid" with 441.4 Mbp on average, one was "DNA triploid" with 670.8 Mbp, and three were "DNA tetraploid" with 879.2 Mbp (1C) on average (Tables 1 and 2). The 1Cx-value was fairly constant between individuals at 441.4 Mbp (CV = 1.6%, $n = 7$, nonsignificant difference between individuals upon two-sided monofactorial ANOVA, $F_{6,13} = 1.09$, $P = 0.418$), and the variances were homogeneous (Levene test, $P = 0.177$). Likewise, different ploidy levels did not differ significantly in their Cx-values ($F_{2,4} = 0.340$, $P = 0.730$).

3.2.2. Flow Cytometry Using Decorticated Twigs. As far as we know, the use of woody plant twigs in FCM for otherwise recalcitrant material is new. The test of interference of

Table 2: Genome sizes of diploid, triploid and tetraploid Icelandic birch: mean 1C-values and average sizes between FDM and FCM.

Ploidy group	1C (Mbp)			Average size in pg (1C)
	FDM	FCM	Average size	
Diploid	441.4	457.6	447.8	0.46
Triploid	670.8	663.8	666.1	0.68
Tetraploid	879.2	883.9	881.8	0.90

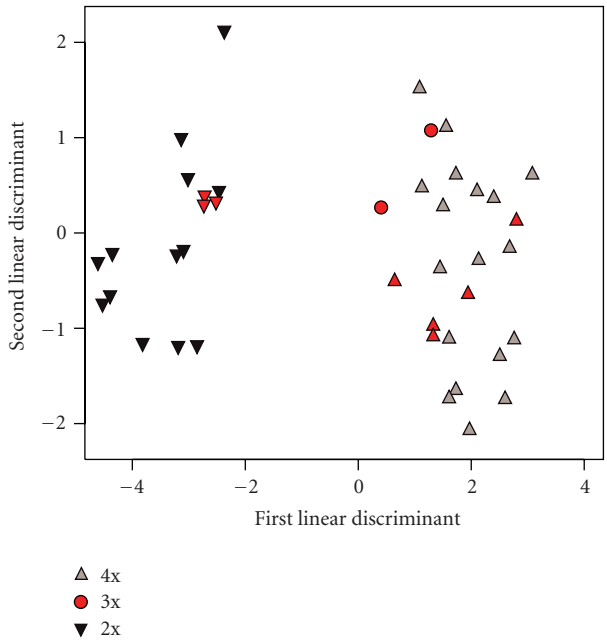

Figure 4: Linear discriminant analysis of leaf shape variables in 41 birch plants from Bifröst: 16 diploid, 2 triploid, and 23 tetraploid. Originally there were 3 triploid plants, but one was missing from this data set although the plant was characterized (Figure 3(b)). The labels in red are individuals used in the genome size study, 10 in total. The analysis shows two clusters, diploid and tetraploid. The triploid plants fall within the tetraploid cluster.

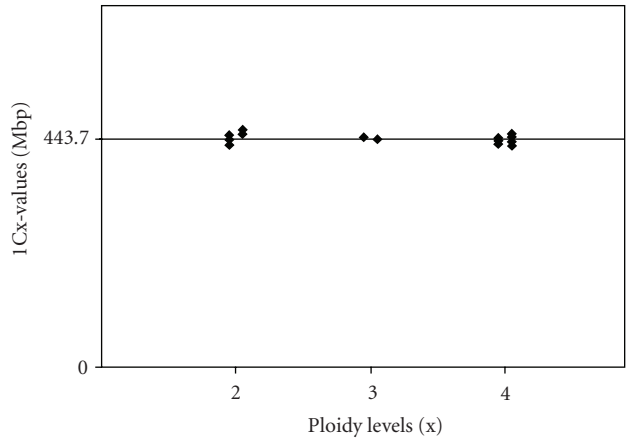

Figure 5: Distribution of 1Cx-values of birch samples from Bifröst woodland according to ploidy levels and techniques (FDM at left, FCM at right). The horizontal line indicates the grand mean of all measurements (see Table 1).

phenolics showed unexpectedly somewhat increased fluorescence and improved peak CVs in both birch and standard, if chopped in combination, compared with lone isolates of both. In five tests, the increase in nuclei fluorescence was 1.043-fold in the standard and 1.038-fold in *Betula pubescens*. This is at present difficult to explain and needs further investigation, but the tests seem to indicate a compensating effect of the joint preparation which justifies the internal standardization.

The results from nine accessions examined clearly disclosed the respective ploidy levels: two were "DNA diploid" with 457.6 Mbp on average, two were "DNA triploid" with 663.8 Mbp, and five were "DNA tetraploid" with 883.9 Mbp (1C) on average (Tables 1 and 2). The ploidy status derived from FCM again corresponded unambiguously with the chromosome number obtained cytogenetically. The two samples that had the growth habit of *B. nana* in the field (no leaves in mid-winter for botanical identification) were correctly identified as belonging to the diploid species (Table 1). The Cx-values of these three ploidy levels were also subjected to ANOVA, but the differences were found to be nonsignificant ($F_{2,6} = 3.048$, $P = 0.122$). However, a weak but real downsizing cannot be rejected and this might be detected in the future using a larger sample size. The mean Cx-value was 0.966-fold in tetraploids compared with diploids, and the intermediate Cx-value in triploids was very close to the theoretical expectation.

3.2.3. Flow Cytometry Compared with Feulgen Densitometry. From the four individuals (one triploid and three tetraploid) measured using both techniques (FDM and FCM), no technique-dependent trend in C-values can be deduced (Table 3). The nonparametric Wilcoxon test for paired samples revealed a two-sided error significance of 0.715, and so both methods yielded equivalent results. All FCM-derived Cx-values compared with FDM-derived ones showed that the individuals in both groups were not statistically different ($df_{1,14}$ $F = 0.894$, $P = 0.360$). The Cx-values of the three ploidy levels obtained with either method (i.e., all $2x$, $3x$, and $4x$ data compared) were also not different ($df_{2,13}$ $F = 0.966$, $P = 0.406$). The overall mean 1Cx-value from all three ploidy groups is 444 Mbp (Figure 5).

In summary, based on the average 1C-values obtained, the 1C genomes of diploid *Betula nana*, triploid interspecific hybrid and tetraploid *B. pubescens* were found to be 448, 666 and 882 Mbp DNA, respectively (Table 2). The mean C-value of the tetraploid species *B. pubescens* was therefore very

Table 3: Comparison of 1Cx-values (Mbp) obtained with FCM and FDM methods.

Plant no.	1Cx (FCM)	1Cx (FDM)	Ratio FCM/FDM
81 (3*x*)	447.2	441.7	1.012
71 (4*x*)	445.4	439.3	1.014
83 (4*x*)	433.7	453.5	0.956
92 (4*x*)	439.7	438.4	1.003
Means 4*x*	439.6	443.7	0.991

close to being twice (1.97-fold) that of the diploid species *B. nana* (Table 2). The mean C-value of the triploid hybrids was intermediate between the two species, very close to the theoretical expectation. The morphological assessment of these plants, on the other hand, did not reveal a linear relationship among the three ploidy groups (Table 1, Figure 4).

4. Discussion

Genome sizes of Icelandic birch obtained in this study correlate perfectly with the ploidy level—the average 1C genome size of the tetraploid *Betula pubescens* (882 Mbp) is about twice the size of the genome of the diploid species *B. nana* (448 Mbp), and the triploid group has the average size of 666 Mbp, which is mid-way between the two species.

The discovery that the triploid hybrid genome is intermediate in its size has a great biological significance. Firstly, it confirms that the triploids are interspecific hybrids of these two coexisting birch species, most probably containing a complete genome from each parental species. Chromosomal mapping in Icelandic birch using the 18S-25S ribosomal markers [1, 30] has also shown that the triploid genome is a hybrid containing one set of chromosomes from each parental species. The triploid genome has five nonhomologous 18S-25S rDNA sites, two from *B. nana* and three from *B. pubescens* [1]. Secondly, the present study indicates that triploid birch in Iceland is probably of recent origin. As the genome size of the triploid group is an almost exact intermediate between the currently introgressed parental species, it must have been formed through recent hybridization. Our palynological study [31] has indeed shown that hybridization in Icelandic birch occurred after the first colonization of birch species in Iceland in the early Holocene and has been going on ever since. Thirdly, there seems to be no major genome/chromosome rearrangements accompanying the interspecific hybridization that could have resulted in significant deviation from the intermediate hybrid genome size observed in the present study. Although we need to measure genome size in a much larger sample size, these first results are statistically significant and hence meaningful. It is impressive that even in the face of extensive gene flow, based on morphological assessment, genome size has proven to be a stable character. There must have been some form of selection pressure that has maintained the species integrity despite introgression. Some elements in the arctic and subarctic environments may have a role in maintaining the genomic stability, by for example, selecting against aneuploidy.

Genome downsizing cannot be statistically proven in the polyploid genomes of these plants. This could be considered a rare situation given that genome downsizing is common in angiosperms [9], whereby polyploids are expected to have proportional larger C-values than their diploid progenitors. The present study shows 1.97x increase in genome size on average, from diploid to tetraploid levels, whereas the analysis of observed genome size changes in angiosperms [9] reveals at the most 1.2x increase for eudicots. The average Cx-values among different ploidies in the present study are also constant statistically. The reason for not seeing genome downsizing in our study could be that the diploid species *B. nana* is probably not the diploid progenitor of the tetraploid *B. pubescens*. The two species are taxonomically and geographically differentiated in a broad sense. In geographically overlapping zones, such as Iceland, the two species hybridize but this secondary contact does not give rise to a new species. Taxonomically, *B. nana* belongs to the subsection *Nanae*, whereas *B. pubescens* belongs to the subsection *Albae* with the other European tree birch species *B. pendula* [28, 32]. The ribosomal mapping on chromosomes of *B. nana* and *B. pubescens* [30] supports the two species having different genome origins. The phylogenetic studies using the nuclear ADH gene and DNA sequences of the chloroplast matK gene [33] as well as AFLP markers [34] indicate that the tetraploid species *B. pubescens* has a hybrid origin, that is, is an allopolyploid. However, no conclusive evidence has been found as to the identity of its diploid progenitors. Until the hybrid genomes of the tetraploid species *B. pubescens* have been correctly identified, it is not possible to test if genome downsizing has actually occurred in *Betula*.

The Plant DNA C-values Database [35] reports 1C-values of only two *Betula* species, an eastern N-American tree birch species *B. populifolia* with 0.2 pg [36] and *B. pubescens* (European tree birch species examined in the present study) with 0.75 pg [37], previously given as 0.7 pg [38]. Both species were measured using Feulgen densitometry after acetic alcohol fixation, without chromosome counts reported. For both species, a diploid chromosome number $2n = 28$ is given in the C-values Database. However, all chromosome counts for *B. pubescens* according to Index to Plant Chromosome Numbers (IPCN, on 16 October 2009, http://mobot.mobot.org/W3T/Search/ipcn.html) and many earlier sources report the tetraploid number $2n = 56$. Apart from this, the differing 1C-values of 0.7 or 0.75 pg for *B. pubescens* [37, 38, resp.,] come from using a recalibrated standard value of *Senecio vulgaris* in Mowforth's thesis [37] (compare Bennett and Smith [39, 40] and see footnotes *b*

and i in the latter). Assuming that the values from [37, 38] actually refer to *B. pubescens* with $2n = 56$, their 1C-value of 0.7 pg or 0.75 pg is 0.78- to 0.83-fold the present 1C-value of 0.902 pg for the tetraploid level. Both these lower Feulgen values are however compatible with our data, if possible tannin interference in their samples, which one could expect in birch after acetic alcohol fixation (see Greilhuber [29]) is assumed. The 1C-value of 0.2 pg for *B. populifolia* [36] appears too low for a diploid birch.

The congruence of FDM- with FCM-values in the present study and their unbiased correlation with ploidy levels agrees with the visual aspect of good, regularly stained formaldehyde-fixed Feulgen-stained nuclei, even though ample solidified tannins were present in many cells of the bud tissues. FDM is clearly a valid method for the purpose. Regarding FCM, the suitability of the chopping method for young decorticated and, in our case, dormant twigs is a useful addition to the flow cytometric practice. First of all, twigs of trees are, it seems, an organ of woody plants that are always available but hitherto never or rarely employed (see Supplemental Appendix in [41]). It is worth noting that in some cases stems of shrubby Fabaceae were used instead of leaves to improve histogram quality [42]. Secondly, this approach extends the application to the cold season, when leaves are not available and buds may be unsuitable because of secondary compounds such as resins. In birch, twig tissue has, in contrast to buds, no disturbing resins. The use of young and even dormant twigs for genome size measurement as reported in this study should be applicable for studying a broad range of tree species.

Acknowledgment

The authors thank I. J. Leitch for communicating details on methods used for Feulgen DNA measurement in *Betula pubescens* from the thesis of M. A. G. Mowforth.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 316356, 7 pages
doi:10.1155/2010/316356

Research Article

Endopolyploidy in Bryophytes: Widespread in Mosses and Absent in Liverworts

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Received 1 March 2010; Accepted 30 April 2010

Academic Editor: Johann Greilhuber

Endopolyploidy occurs when DNA replication is not followed by mitotic nuclear division, resulting in tissues or organisms with nuclei of varying ploidy levels. Endopolyploidy appears to be a common phenomenon in plants, though the prevalence of endopolyploidy has not been determined in bryophytes (including mosses and liverworts). Forty moss species and six liverwort species were analyzed for the degree of endopolyploidy using flow cytometry. Nuclei were extracted in LB01 buffer and stained with propidium iodide. Of the forty moss species, all exhibited endopolyploid nuclei (mean cycle value = 0.65 ± 0.038) except for the *Sphagnum* mosses (mean cycle value = 0). None of the liverwort species had endopolyploid nuclei (mean cycle value = 0.04 ± 0.014). As bryophytes form a paraphyletic grade leading to the tracheophytes, understanding the prevalence and role of endopolyploidy in this group is important.

1. Introduction

Polysomaty is the occurrence of nuclei of varying ploidy levels in the same individual, often associated with different cell or tissue types. This condition of nuclei of varying ploidy levels, known as endopolyploidy, is a result of endoreduplication, which occurs when DNA replication is not followed by mitosis. The mechanisms behind endoreduplication are suggested to involve changes in the activity of cyclin-dependent kinases, which affect the normal transition of the cell cycle [1]. There is, however, a lack of knowledge and understanding regarding the extent, role, and control of endopolyploidy in plants [2].

Various hypotheses have been suggested to explain the importance of endopolyploidy, including growth, development, and stress response [1, 3–5]. One suggested role of endopolyploidy relates directly to the "Nucleotypic Theory," which states that DNA content directly impacts cell volume and other phenotypic traits, which in turn affects various aspects of organism form and function [6, 7]. Barow and Meister [8] and Jovtchev et al. [9] have produced evidence to support this hypothesis, finding that endopolyploidy can allow plants with small genomes to have increased nuclear and cell volume to assist in growth and development. In turn, endopolyploidy is correlated with life history strategy and phylogenetic affiliation [8] and is influenced by various environmental factors including temperature [10, 11], light [12], drought [13], and salinity [14].

Among land plants, endopolyploidy is common in angiosperms but appears to be rare in gymnosperms and ferns [15]. According to a summary completed in 2007, out of thirty explored angiosperm families, nineteen families contain species that predominantly exhibit endopolyploidy [16]. Endopolyploidy occurs in various algal groups [17–19], but in gymnosperms, endopolyploidy is scarce [8, 20], and in ferns there are only isolated references [21–23].

In bryophytes (broadly referring to mosses, liverworts and hornworts), the frequency of endopolyploidy is not known, though some studies present data on specific species or specifically targeted tissues. These studies include the presence of endopolyploidy in polytrichaceous mosses including food-conducting cells [24] and mucilaginous hairs and parenchyma [25], and endopolyploid caulonema in *Funaria hygrometrica* [26, 27]. Endopolyploidy has also been

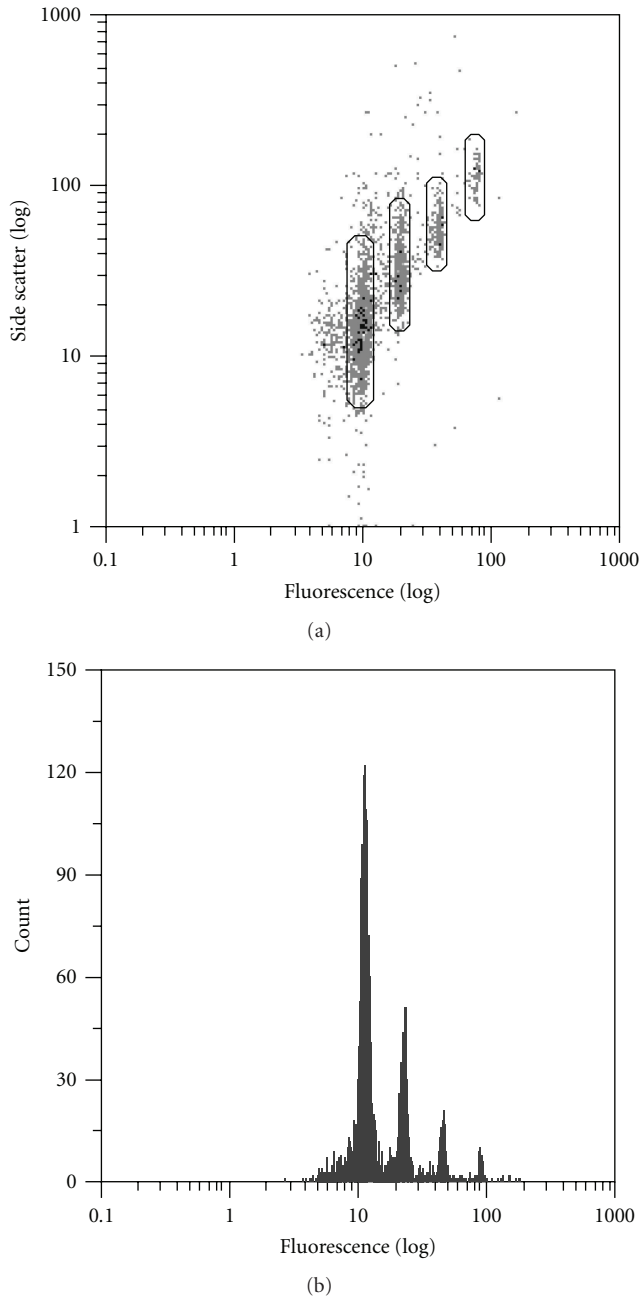

Figure 1: Determination of endopolyploidy in *Rhytidiadelphus triquetrus* using flow cytometry. (a) Scattergram of side scatter versus fluorescence with polygon gates. (b) Histogram of counts versus fluorescence.

observed in moss rhizoids and food conducting tissues [28]. The use of flow cytometry to observe endopolyploidy in bryophytes has been referred to anecdotally [29]. Additionally, endopolyploidy has been explored in the model moss, *Physcomitrella patens*, which has a unique case of exhibiting two distinct ploidy levels in different tissues. Chloronema cells were found to have predominantly 2C or G2 nuclei, while the caulonema nuclei were 1C [30]. In an initial assessment of the *P. patens* genome, there were so few nuclei in the 1C phase; the 2C peak was mistakenly identified as the 1C peak [31]. Treatment of *P. patens* with auxin resulted in an increase in 1C nuclei and also an increase in 4C nuclei [32]. Older caulonema cells also had a higher degree of endopolyploidy [33].

As bryophytes represent the earliest plants to inhabit terrestrial ecosystems [34], the role of endopolyploidy in this group of organisms is relevant in order to increase our understanding of the evolution of endopolyploidy. Bryophytes have small genome sizes [35] and exhibit unique life history strategies [36, 37] as well as habitat specificity [38, 39]. Flow cytometry provides an efficient way to observe endopolyploidy over a range of specimens. These factors make bryophytes ideal organisms to explore the prevalence, role, and biological significance of endopolyploidy. The objective of the present study is to provide the first survey of the prevalence of endopolyploidy in bryophytes.

2. Materials and Methods

Bryophyte specimens were collected in Ontario, Canada, in the summer of 2009. Forty moss species representing seventeen families and six liverwort species from five families were collected (see Table 1). Voucher specimens are deposited in the Biodiversity Institute of Ontario Herbarium (OAC/BIO), University of Guelph. From each population, three independent replicates were analyzed on separate days using flow cytometry, except for three of the liverwort species, where there was insufficient tissue. The samples were composed of green shoots, which included both stem and leaf material. General methodology followed Galbraith et al. [40] and Doležel et al. [41] and was refined according to Bainard et al. [42]. Approximately 10mg of air-dried bryophyte tissue was chopped in 1.2 ml cold LB01 buffer and the resulting solution was filtered through a 30 μm mesh. The nuclei were stained with 150 $\mu g\,ml^{-1}$ propidium iodide (Sigma) in the presence of 0.5 $\mu g\,ml^{-1}$ RNase A (Sigma). Samples were incubated on ice for 20 minutes. For each sample, at least 1000 nuclei were analyzed.

Flow cytometric analysis was completed on a Partec CyFlow SL (Partec GmbH, Münster, Germany) equipped with a blue solid-state laser tuned at 20 mW and operating at 488 nm. Before each use, the instrument was calibrated using 3 μm calibration beads (Partec, Münster, Germany). The parameters recorded for each bryophyte sample included fluorescence intensity at 630 nm measured on a log scale, forward scatter and side scatter. These parameters were observed alone and in combined scattergrams including: fluorescence versus side scatter and fluorescence versus forward scatter.

To determine the degree of endopolyploidy, the number of nuclei (n) in each ploidy level was counted, using FloMax Software by Partec (Version 2.52, 2007). Due to the interference of debris particles, polygon gates were drawn around the nuclei of interest on the fluorescence versus side scatter scattergram to determine the number of nuclei in each peak (Figure 1). To quantify the degree of endopolyploidy, the cycle value was calculated, which is a measure of the number

Table 1: Degree of endopolyploidization of forty moss species and six liverwort species. Mean cycle value and mean *C*-level results are based on three replicates except where noted.

Family	Species	Mean Cycle Value ± Standard Error	Mean *C*-level ± Standard Error
Mosses			
Sphagnaceae	*Sphagnum angustifolium*	0.00 ± 0.000	1.00 ± 0.000
	Sphagnum recurvum	0.00 ± 0.000	1.00 ± 0.000
Polytrichaceae	*Polytrichum commune*	0.33 ± 0.055	1.39 ± 0.068
	Polytrichum juniperum	0.60 ± 0.060	1.80 ± 0.090
Fissidentaceae	*Fissidens taxifolius*	0.40 ± 0.039	1.48 ± 0.056
Dicranaceae	*Dicranum condensatum*	0.87 ± 0.072	2.17 ± 0.148
	Dicranum flagellare	0.50 ± 0.015	1.62 ± 0.022
	Dicranum fuscescens	0.57 ± 0.087	1.75 ± 0.125
	Dicranum montanum	0.52 ± 0.029	1.63 ± 0.041
	Dicranum polysetum	1.11 ± 0.030	2.34 ± 0.049
	Dicranum scoparium	0.98 ± 0.048	2.17 ± 0.101
	Trematodon ambigus	0.51 ± 0.055	1.62 ± 0.063
Ditrichaceae	*Ceratodon purpureus*	0.51 ± 0.060	1.73 ± 0.067
Orthotrichaceae	*Orthotrichum speciosum*	0.29 ± 0.071	1.40 ± 0.114
Hedwigiaceae	*Hedwigia ciliata*	0.34 ± 0.030	1.51 ± 0.056
Aulacomniaceae	*Aulacomnium androgynum*	0.64 ± 0.076	1.91 ± 0.092
Mniaceae	*Plagiomnium drummondii*	1.37 ± 0.054	2.91 ± 0.105
	Plagiomnium medium	1.21 ± 0.152	2.81 ± 0.353
	Pohlia whalenbergia	1.13 ± 0.125	3.33 ± 0.282
Hylocomiaceae	*Hylocomnium splendens*	0.53 ± 0.165	1.71 ± 0.253
	Pleurozium schreberi	0.35 ± 0.079	1.42 ± 0.098
	Rhytidiadelphus triquetrus	0.52 ± 0.036	1.75 ± 0.069
Leskeaceae	*Haplocladium microphyllum*	0.41 ± 0.086	1.55 ± 0.117
Thuidiaceae	*Thuidium delicatulum*	0.91 ± 0.119	2.30 ± 0.184
	Thuidium minulatum	0.41 ± 0.025	1.52 ± 0.032
Campyliaceae	*Campylium chrysophyllum*	0.64 ± 0.057	1.77 ± 0.077
Brachytheciaceae	*Brachythecium acuminatum*	0.54 ± 0.045	1.65 ± 0.049
	Brachythecium salebrosum	0.14 ± 0.018	1.16 ± 0.023
	Brachythecium velutinum	0.51 ± 0.071	1.69 ± 0.116
	Eurhynchium pulchellum	0.27 ± 0.044	1.36 ± 0.066
Plagiotheciaceae	*Plagiothecium denticulatum*	1.05 ± 0.160	2.51 ± 0.282
	Plagiothecium laetum	1.70 ± 0.062	4.01 ± 0.243
Climaciaceae	*Climacium dendroides*	1.48 ± 0.030	3.40 ± 0.086
Hypnaceae	*Callicladium halandianum*	0.69 ± 0.187	1.89 ± 0.246
	Hypnum curvifolium	1.29 ± 0.107	3.03 ± 0.226
	Hypnum lindbergii	0.78 ± 0.236	2.11 ± 0.290
	Hypnum pallescens	0.96 ± 0.078	2.77 ± 0.160
	Hypnum recurvatum	0.34 ± 0.104	1.46 ± 0.144
	Ptilium crista-castrensis	0.27 ± 0.016	1.42 ± 0.018
	Pylaisiella polyantha	0.37 ± 0.054	1.43 ± 0.072
	Mean	**0.65 ± 0.038**	**1.94 ± 0.065**
Liverworts			
Ptilidiaceae	*Ptilidium pulcherrimum*	0.00 ± 0.000	1.00 ± 0.000
Geocalycaceae	*Lophocolea heterophylla*	0.09*	1.06*
Calypogeiaceae	*Calypogeia integristipula*	0.12 ± 0.016	1.12 ± 0.016
Jungermanniaceae	*Barbilophozia barbata*	0.01 ± 0.008	1.08 ± 0.008
	Lophozia heterocolpos	0.06*	1.06*
Radulaceae	*Radula complanata*	0.02** ± 0.008	1.02** ± 0.008
	Mean	**0.043 ± 0.014**	**1.04 ± 0.014**

* Value based on one replicate.
** Mean based on two replicates.

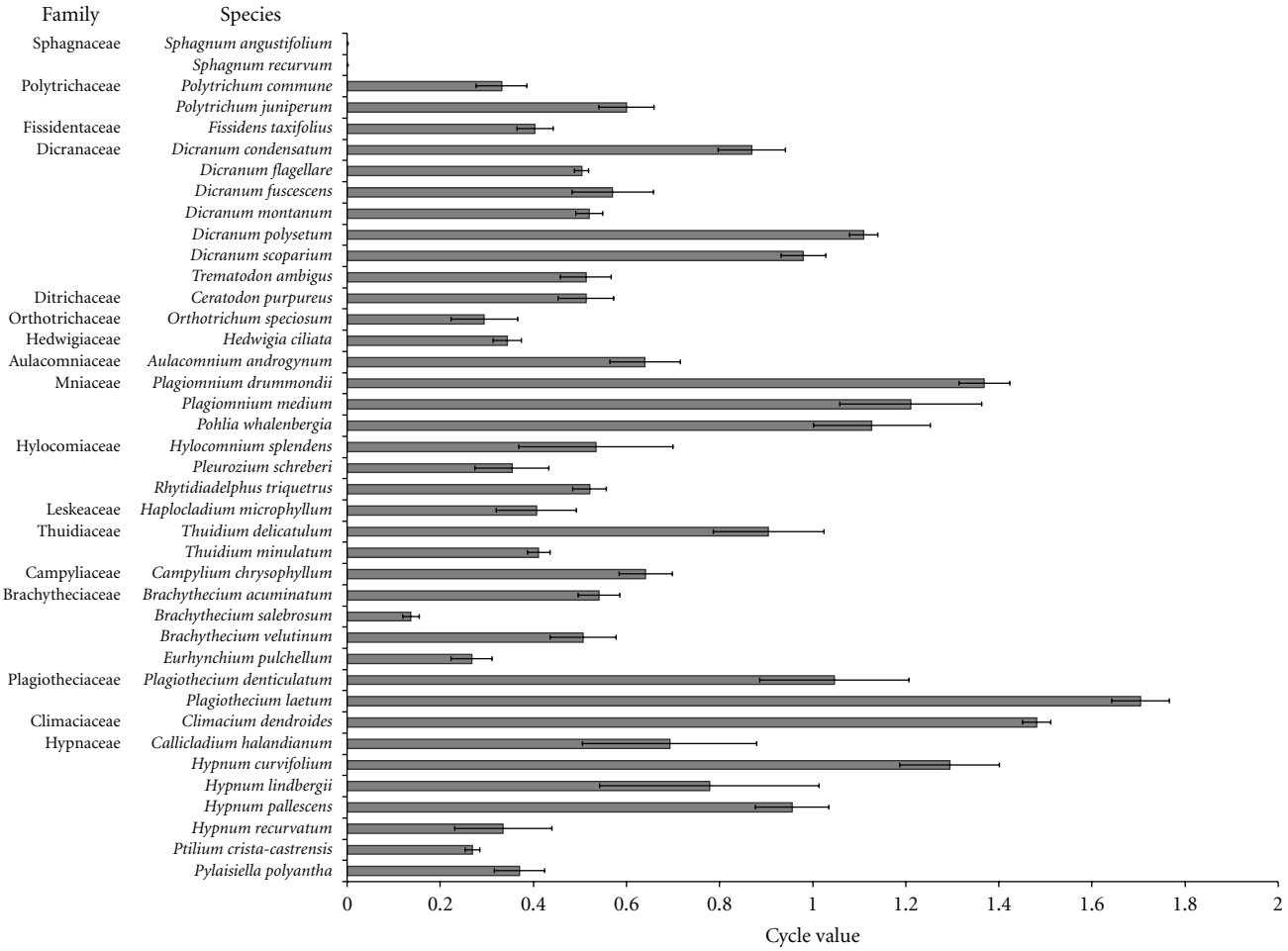

FIGURE 2: Mean cycle value of 40 moss species, determined using flow cytometry. Error bars represent standard error of the mean ($n = 3$).

of endoreduplication cycles per nucleus that occurred in the nuclei measured [8]. As bryophytes are haplophasic, the first endopolyploid level is the 2C level, which corresponds to one endoreduplication cycle. This is calculated according to the following [16]:

$$\text{Cycle value} = \frac{(0 \times n_{1c} + 1 \times n_{2c} + 2 \times n_{4c} + 3 \times n_{8c} + 4 \times n_{16c} \cdots)}{(n_{1c} + n_{2c} + n_{4c} + n_{8c} + n_{16c} \cdots)}. \quad (1)$$

Additionally, the mean C-level was calculated, which is a measure of the mean ploidy level of the nuclei measured [10, 43]. This is calculated using the following [16]:

$$\text{Mean } C\text{-level} = \frac{(1 \times n_{1c} + 2 \times n_{2c} + 4 \times n_{4c} + 8 \times n_{8c} + 16 \times n_{16c} \cdots)}{(n_{1c} + n_{2c} + n_{4c} + n_{8c} + n_{16c} \cdots)}. \quad (2)$$

It should be noted that small amounts of nonendopolyploid nuclei can contribute to the number of nuclei in the different ploidy levels. For example, nuclei that were in the G2 phase of the cell cycle would have a 2C ploidy level, and not necessarily be endoreduplicated nuclei. As well, nuclei can occasionally stick together (forming doublets) and contribute to higher ploidy levels. However, it is expected that in most cases the relative amount of G2 and doublet nuclei will be negligible [16]. Additionally, species with a cycle value less that 0.1 are not considered to be endopolyploid [8, 9].

3. Results

All moss species measured in this study had distinctly endopolyploid nuclei, with the exception of the *Sphagnum* mosses (Table 1 and Figure 2). Examples of the flow cytometry results are shown in Figure 3. The average cycle value over all mosses was 0.65 ± 0.038 and the mean C-level was 1.94 ± 0.065. Other than the *Sphagnum* species, all mosses had 1C, 2C and 4C nuclei present, and several also had 8C and 16C nuclei. The bryophyte with the highest degree of endopolyploidy was *Plagiothecium laetum*, with a mean cycle value of 1.71 and a mean C-level of 4.01 (see Figure 3).

In contrast, the liverworts we sampled had almost no endopolyploid nuclei (Table 1). The mean cycle value for

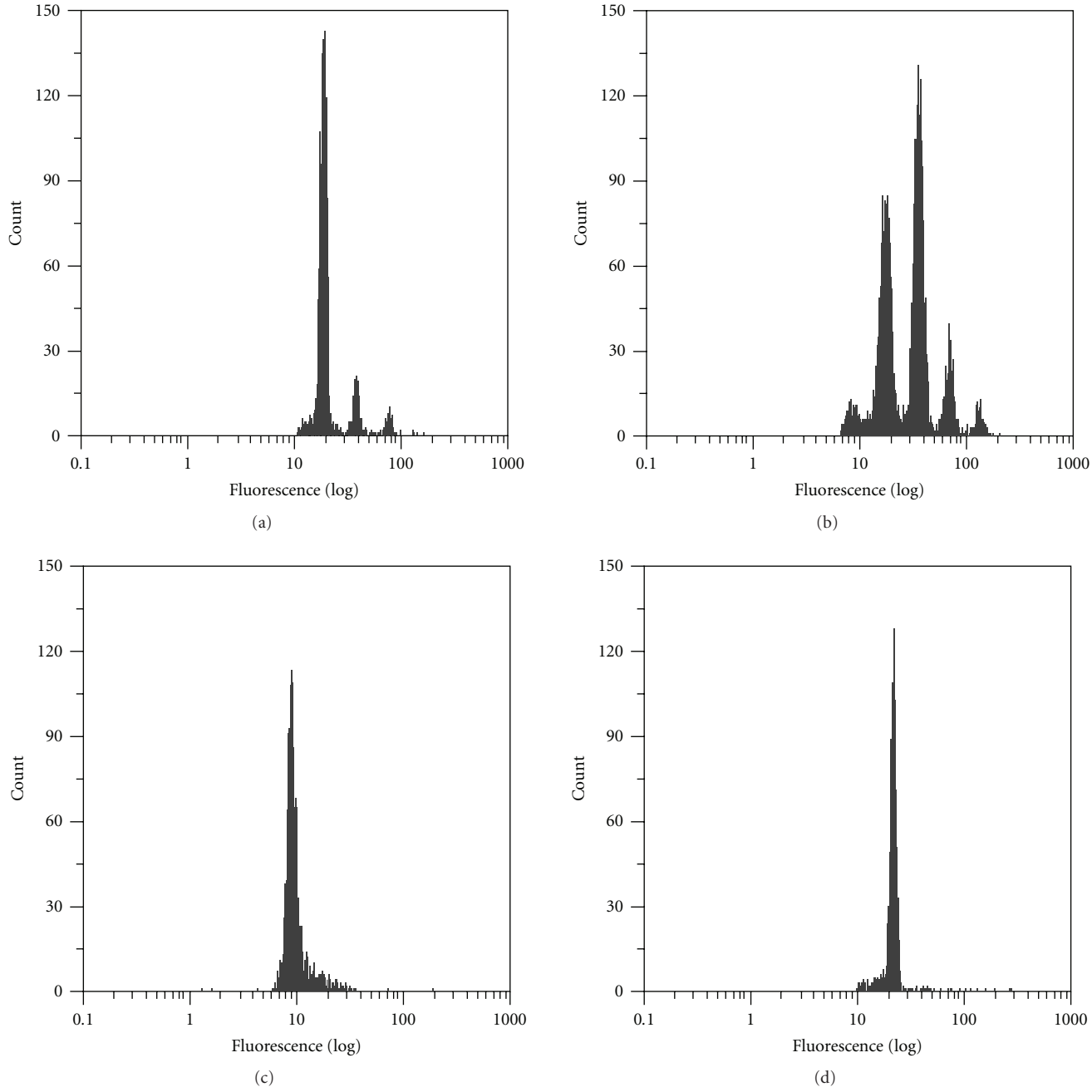

Figure 3: Examples of fluorescence histograms for several species. (a) *Brachythecium salebrosum*, showing a low degree of endopolyploidization (cycle value = 0.14 ± 0.018). (b) *Plagiothecium laetum,* exhibiting the highest degree of endopolyploidization (cycle value = 1.70 ± 0.062). (c) *Sphagnum recurvum*, exhibiting no endopolyploidization. (d) *Barbilophozia barbata* (liverwort), exhibiting no endopolyploidization.

the liverworts was 0.04 ± 0.014 and the mean *C*-level was 1.04 ± 0.014. Only *Calypogeja integristipula* had a cycle value over 0.1, and this was most likely due to the presence of diploid sporophytes in the population, which were difficult to remove at the time of processing. All other species had cycle values below 0.1, which indicates that if there were nuclei in a second peak, they were likely G2 or doublet nuclei.

4. Discussion

Endopolyploidy appears to be widespread in mosses, and absent in liverworts. The species coverage in the current study is not large enough to make conclusions regarding the phylogenetic affiliation of endopolyploidy in bryophytes, however general comments can be made. Some of the moss families analyzed appeared to have a higher incidence of

endopolyploidy than others, such as the Mniacea. Other families showed considerable variation between species, such as the Dicranaceae and Hypnaceae. The lack of endopolyploidy in the Sphagnaceae could be attributed to the unique occurrence of a large proportion of dead cells (large hyaline cells) to small, green, living cells (chlorophyllose cells) within the leaves [44]. Greater species coverage will allow a more comprehensive view of the prevalence of endopolyploidy in relation to taxonomy.

It is interesting that the liverworts sampled are lacking in endopolyploid nuclei, even though they are closely related to the mosses. Although liverworts have similar life history strategies to mosses, there are considerable biological differences that include: short-lived sporophytes that wither away not long after releasing spores; single-celled rhizoids; the lack of clearly differentiated stem and leaves in thallose species; the presence of deeply lobed or segmented leaves arranged in three ranks; and the presence of oil bodies in at least some of their cells, which are absent from most other bryophytes and from all vascular plants [45]. The disparity in the degree of endopolyploidization between mosses and liverworts could be related to these morphological and biological differences.

From a phylogenetic perspective, as liverworts are sister to all land plants [46] and appear to have a low occurrence of endopolyploid nuclei, endopolyploidy is likely a derived trait. Additionally, the lack of endopolyploidy in Sphagnaceae suggests that the trait evolved after this divergence in bryophytes. Endopolyploidy has likely evolved independently in various groups, as angiosperm families also have varying degrees of endopolyploidy. Future research should involve a broader species coverage across land plants to better understand the phylogenetic implications of endopolyploidy.

As the biological significance of endopolyploidy is just beginning to be explored, there is a considerable amount still to be discovered in relation to bryophyte morphology and environment. It is necessary to determine the cells and tissues responsible for the varying DNA contents, in order to understand the biological role that endopolyploidy plays in bryophyte form and function. Additionally, the environmental impact on endopolyploidization will be especially relevant as bryophytes exhibit habitat specificity. We are currently conducting a more comprehensive survey of the prevalence of endopolyploidy in hepatics, and exploring hypotheses concerning the relative frequency of endopolyploidy (particularly in mosses) in a group of plants that are sister to tracheophytes [46].

As genome size and endopolyploidy appears to be correlated [8], the small genome sizes of mosses [35] and the high degree of endopolyploidy in this group seem to fit this trend. However, this relationship should be explored further, and determination of genome size for the bryophyte species mentioned here is already underway by our research group. Understanding genome size in relation to endopolyploidy and relating DNA content to cell size and function in bryophytes will continue to elucidate the biological significance of endopolyploidy.

Acknowledgments

The authors would like to thank three anonymous reviewers and Johann Greilhuber for critical review of our manuscript. Many thanks go to Aron J. Fazekas for feedback and consultation and to Jose Maloles, Kelsey O'Brien, and Benjamin Yim for assistance in the field and lab. This work was supported by the National Science and Engineering Research Council of Canada (PGS D to J.D. Bainard; CRD to S.G. Newmaster) and the Canadian Foundation for Innovation (LOF to S.G. Newmaster).

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 930895, 9 pages
doi:10.1155/2010/930895

Research Article

Leaves and Seeds as Materials for Flow Cytometric Estimation of the Genome Size of 11 Rosaceae Woody Species Containing DNA-Staining Inhibitors

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Received 31 December 2009; Revised 20 February 2010; Accepted 1 April 2010

Academic Editor: João Loureiro

The presence of some secondary metabolites in the cell cytosol can cause a stoichiometric error in the flow cytometric estimation of nuclear DNA content. There is no fully reliable method to completely eliminate the effect of these compounds on nuclei fluorescence, and therefore using plant organs/parts free of staining inhibitors is recommended. Eleven species of Rosaceae with high concentrations of propidium-iodide-staining inhibitors were studied to check the possibility of using seeds instead of leaves for genome size estimation. Despite optimizing the concentration and composition of antioxidants in nuclei-isolation buffer for each species, the effect of cytosolic compounds present in the leaves could not be avoided entirely. None of the seeds of the studied species contained inhibitors, and they produced histograms of good quality. The genome size of the studied species ranged from 1.15 to 3.17 pg/2C; for 10 species the DNA content was estimated for the first time.

1. Introduction

The Rosaceae family is economically very important because many of the species are cultivated for their fruits (e.g., *Malus*, *Pyrus*, *Prunus*, *Fragaria*, and *Rubus*) or have ornamental value (*Rosa*). It has been well studied, although the systematic position and evolution of many taxa are still not clear. Knowledge of the genome size would be helpful in their classification. Within the Rosaceae, polyploidy series from diploid to 12-ploid or higher occur [1, 2]. Since the chromosomes are small and often numerous, ploidy estimation by chromosome counts is difficult. In addition, microscopic chromosome counting is time-consuming and limited to a few tissues. Therefore, flow cytometry (FCM) is a more convenient alternative for establishing the ploidy/genome size of Rosaceae species.

Flow cytometry, a fast and accurate method for the estimation of DNA content, has become the predominant technique for establishing plant genome size [3, 4]. The most common procedure of sample preparation involves chopping plant organs/tissues (mainly leaves) in a nuclei-isolation buffer and measuring the fluorescence of a fluorochrome intercalated into the DNA double helix [5]. The composition of the isolation buffer is critical for accurate FCM measurements. Besides facilitating the isolation of intact nuclei, it should maintain their stability, prevent their aggregation, protect DNA from degradation, and provide an appropriate environment for specific and stoichiometric staining of nuclear DNA [6]. During the last decade, one of the most challenging problems faced by FCM users has been adjusting the buffer composition to the requirements of specific species, especially if they contain staining inhibitors such as phenols, caffeine, and other secondary metabolites in the cytosol of their leaf cells [6–10]. Many species of the Rosaceae family, especially woody plants, belong to this "difficult" group [1, 11, 12]. Special and more time-consuming procedures have been applied to analyze their DNA content by flow cytometry, involving two-step nuclei extraction and a staining protocol, and/or optimizing buffer composition by the addition of antioxidants [1, 2, 13–15].

As shown before [6, 10, 11, 16] and in the present paper, the buffer composition as well as the antioxidant choice and concentration should be optimized individually, which involves additional, often multiple and time-consuming steps before genome size estimation. Omitting such steps can result in inaccurate estimation of the nuclear DNA content. Thus, a simple procedure for sample preparation of these difficult species would speed up analysis of their genome size as well as of the genome composition of allopolyploid/hybrid species.

Despite many studies, there is little information on the mode of action of the staining inhibitors and no universal method to completely avoid their effects on DNA content estimation ([4, 17] and references therein). Inhibition probably involves the intercalation of secondary metabolites into DNA and/or their direct reaction with the dye molecule that interferes with its fluorescence [7, 10]. The addition of antioxidants, such as polyvinylpyrrolidone, β-mercaptoethanol, or dithiothreitol to a buffer, although helpful, does not always guarantee the correct measurement of DNA content, especially if added in too low concentration ([11, 18], this study). Since the composition and concentration of secondary metabolites is different in different species/tissues, even buffers currently developed for "difficult" species; for example, woody plant buffer (WPB: [6]) cannot be considered as suitable for all of them. Moreover, for the leaves or even leaf buds of some species that contain a lot of secondary metabolites or/and mucilage, flow cytometry has yielded largely uninformative results (e.g., *Polystachia*, *Ulmus*, *Betula*, *Thymus, Rhododendron, Drosera, Viburnum* [17, 19, 23], Jedrzejczyk and Sliwinska, unpublished results). Therefore, using a plant organ/part that is free of inhibitors is a better alternative for genome size estimation. For example, for *Betula*, young, winter-dormant twigs have been utilized [19]. Our previous reports showed that leaves can be replaced by seeds for genome size estimation [18, 20, 21]. The mature embryos of orthodox seeds (e.g., seeds that acquire desiccation tolerance during development and may be stored in the dry state) usually contain most of their cells arrested in the G_0/G_1 phase of the cell cycle (for review see [22]) and thus are suitable for establishing the 2C-value. The presence of nuclei of different ploidies (endosperm or endoreduplicated embryo nuclei) in some seeds can be overcome by the isolation and utilization of a part where this has not occurred (usually the radicle).

It is generally assumed that DNA content estimates by flow cytometry become more reliable as histogram quality improves, with CV and debris measures typically regarded as the best measures of quality [3, 6, 23]. Previous reports [18] have shown that DNA content measures may differ between seeds and leaves but we present for the first time an analysis of the relative sample quality of seed and leaf tissue. The Roseaceae was selected for this study to test the idea that seeds may be a good alternative to leaves in difficult plant species.

The aim of the present study was to find if the seeds of the 11 Rosaceae woody species containing staining inhibitors in the leaf cytosol are free of such compounds, to compare the quality of the FCM histograms of the leaf and seed nuclei, and determine the genome size of those two materials to check their suitability. We tried to neutralize the effect of the staining inhibitors present in leaves by addition of antioxidants, in concentration and composition established individually for each species. However, in most cases this did not improve the histogram quality parameters to the level of those of the inhibitor-free tissue of the seeds. The effect of the antioxidants on propidium iodide (PI) fluorescence of the nuclei isolated from inhibitor-free tissue (seeds) was studied also. We suggest that using seeds allows the application of a standard one-step protocol to prepare a nuclear sample, without the necessity for time-consuming optimization of buffer composition and the risk of incomplete suppression of inhibitor activity by antioxidants. To the best of our knowledge, for 10 out of the 11 species this also is the first report on their genome size.

2. Materials and Methods

2.1. Plant Material. Nuclear DNA content was estimated in the leaves and seeds of 11 species of trees and shrubs belonging to the Rosaceae family (Table 1). All the species except *Prunus padus* (Prunoidae; $x = 8$) belong to the Spiraeoideae ($x = 17$) subfamily. Plant material (depending on availability, collected from one to three specimens of each species) originated from the Botanical Garden of the Kazimierz Wielki University in Bydgoszcz, Poland. Each studied specimen is recorded in the garden database. Fruits were collected in October-November 2007 and 2008; seeds were removed, dried, and stored at 4°C until the following summer, to be analyzed at the same time as the leaves. Young, fully developed leaves were collected in August and analyzed within 2-3 days; during this period they were stored in humid filter paper at 4°C. *Petunia hybrida* (P × Pc6; 2.85 pg/2C: [24]) and *Zea mays* (CE-777; 5.43 pg/2C: [25]) were used as internal standards.

The whole seed (including seed coat) of *Amelanchier stolonifera*, *Crataegus coccinea, Malus floribunda, P. padus*, and *Sorbus intermedia*, half seed with the radicle (including seed coat) for all species of *Cotoneaster* genus, and the radicle of *Pyrus elaeagrifolia* were used to prepare flow cytometric samples. For internal standards, a single radicle tip (*Z. mays*) or 30–40 whole seeds (*P. hybrida*) were co-chopped with the target species seed or seed part. For species of the *Cotoneaster* genus, 2 or 3 leaves were included in a sample, and for the other species, and the internal standards, leaf blade fragments of about 0.5–1 cm^2 were included. Single samples contained either nuclei from leaves or from seeds of the target species and the internal standard.

2.2. Flow Cytometry. The test for the presence of PI-staining inhibitors in the leaves and seeds of all species was performed following the protocol of Price et al. [7]. For nuclei isolation, Galbraith's buffer [5], supplemented with PI (50 μg/mL) and ribonuclease A (50 μg/mL), was used. PI fluorescence of two samples was compared: sample 1 that contained nuclei isolated from the leaf/leaves or the radicle tip/seeds of an internal standard (*Z. mays* or *P. hybrida*, depending on the species: Table 2), and sample 2 that contained nuclei

Table 1: Presence of staining inhibitors in leaves and seeds of trees and shrubs belonging to the Rosaceae family. SH: shrub; TR: tree.

Species name		Life habit	Presence of inhibitors	
Latin	Common		Leaves	Seeds
Amelanchier stolonifera Wieg.	Running Serviceberry	SH	+	—
Cotoneaster dammeri "Royal Carpet"	Bearberry Cotoneaster	SH	+	—
Cotoneaster divaricatus Rehd. et Wils.	Spreading Cotoneaster	SH	+	—
Cotoneaster hjelmqvistii Flinck & Hylmö	Hjelmqvist's Cotoneaster	SH	+	—
Cotoneaster horizontalis Decne. "Variegatus"	Rockspray (Herring Bones) Cotoneaster	SH	+	—
Cotoneaster veitchii G. Klotz	Many-flowered Cotoneaster	SH	+	—
Crataegus coccinea L.	Scarlet Hawthorn	TR	+	—
Malus floribunda Sieb. ex Van Houtte	Japanese Crabapple	TR	+	—
Prunus padus L.	Bird Cherry	TR	+	—
Pyrus elaeagrifolia Pall.	Oleaster-leafed Pear	TR	+	—
Sorbus intermedia (Ehrh.) Pers.	Swedish Whitebeam	TR	+	—

Table 2: 2C DNA content of leaves and seeds of trees and shrubs belonging to the Rosaceae family.

Species name	Internal standard*	Buffer**	2C DNA (pg, mean ± SD)	
			Leaves	Seeds
Amelanchier stolonifera	2	B	2.59 ± 0.02 a***	2.55 ± 0.01 b
Cotoneaster dammeri	2	C	1.41 ± 0.01^{ns}	1.41 ± 0.02
Cotoneaster divaricatus	2	C	2.78 ± 0.01 a	2.73 ± 0.02 b
Cotoneaster hjelmqvistii	2	C	2.74 ± 0.03 a	2.70 ± 0.01 b
Cotoneaster horizontalis	2	C	2.77 ± 0.03^{ns}	2.77 ± 0.02
Cotoneaster veitchii	2	C	2.67 ± 0.02 a	2.63 ± 0.02 b
Crataegus coccinea	2	B	3.16 ± 0.02^{ns}	3.17 ± 0.02
Malus floribunda	1	B	1.45 ± 0.01^{ns}	1.44 ± 0.01
Prunus padus	1	B	1.15 ± 0.02^{ns}	1.15 ± 0.02
Pyrus elaeagrifolia	1	A	1.15 ± 0.01^{ns}	1.15 ± 0.02
Sorbus intermedia	2	B	2.82 ± 0.01^{ns}	2.81 ± 0.02

*1: *Petunia hybrid*; 2: *Zea mays*'; **A: Galbraith's + 1% (w/v) PVP-10; B: Galbraith's + 1.5% (w/v) PVP-10; C: Galbraith's + 2% (w/v) PVP-10 + 15 mM β-mercapthoethanol; ***2C-values in leaves and seeds of the certain species (in lines) followed by different letters are significantly different at $P = .05$ (Student's t-test); ns: no significant difference.

released simultaneously from the leaf or seed(s)/seed part of a target species and an internal standard. To prepare a sample, selected plant parts were chopped with a sharp razor blade in a plastic Petri dish with 1 mL of the buffer. The suspension was passed through a 50 μm mesh nylon filter. Samples prepared from leaves were analyzed after about 10 minutes of incubation on ice, and samples prepared from seeds after 20–30 minutes [18]. For each sample, fluorescence in at least 7000 nuclei (across all peaks) was measured using a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer, equipped with a high-grade solid-state laser with green light emission at 532 nm, long-pass filter RG 590 E, DM 560 A, as well as with side (SSC) and forward (FSC) scatters. Analyses were performed on five replicates. Histograms were analyzed using the FloMax (Partec GmbH, Münster, Germany) software.

For establishing the concentration and combination of antioxidants that stabilize PI fluorescence in leaf samples, a test for inhibitors was repeated using Galbraith's buffer with the addition of 1% (w/v) polyvinylpyrrolidone (PVP-10; buffer A). For samples from species which after application of buffer A still showed decreased fluorescence of the nuclei of the internal standard, the concentration of PVP in the buffer was increased to 1.5% (w/v) (buffer B) and the inhibitor test performed again. For *Cotoneaster* species, which still showed the inhibition effect on PI fluorescence when buffer B was applied, the combination of 2% (w/v) PVP and 15 mM β-mercaptoethanol (buffer C) was applied. A sample was

Table 3: Effect of antioxidants on flow cytometric estimation of 2C DNA content in seeds of selected species belonging to the Rosaceae family.

Species name	2C DNA (pg, mean ± SD)	
	Buffer without antioxidants	Buffer with antioxidants*
Amelanchier stolonifera	2.56 ± 0.01^{ns}	2.55 ± 0.01
Cotoneaster divaricatus	2.74 ± 0.01^{ns}	2.73 ± 0.01
Crataegus coccinea	3.18 ± 0.02^{ns}	3.17 ± 0.01
Malus floribunda	1.45 ± 0.01^{ns}	1.44 ± 0.00
Prunus padus	1.15 ± 0.01^{ns}	1.15 ± 0.01

*For a certain species the buffer as stated in Table 2 was applied; ns: no significant difference between the values in lines (Student's t-test, $P = .05$).

prepared and analyzed as described above. Analyses were performed on five replicates.

An additional test was performed to study the effect of the presence of antioxidant(s) in a buffer on the estimation of the 2C-value in seeds of selected species (Table 3). The nuclei were isolated using Galbraith's buffer without any antioxidant and buffer supplemented with antioxidant/combination of two antioxidants suitable for the certain species, as established in the previous experiment (Table 2). Measurements were performed on five replicates, using the same procedure of sample preparation and analysis as in the previous two experiments. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of the target species/internal standard on the histogram of fluorescence intensities.

For measurements of genome size in the leaves and seeds, selected plant parts of the target species and of the internal standard were chopped simultaneously using buffer A, B, or C (Table 2). Analyses were performed on 10 replicates, using the same procedure as in the previous experiments. In addition to nuclear DNA content, a debris background factor (DF) before the application of signals gating was calculated according to the following equation [10]:

$$\mathrm{DF} = \frac{\text{Total number of particles} - \text{Total number of intact nuclei}}{\text{Total number of particles}} \times 100\%. \quad (1)$$

The results were estimated using a one-way analysis of variance and a Student's t-test ($P = .05$).

3. Results and Discussion

Many species, especially woody and medicinal plants, produce numerous secondary metabolites that interfere with the staining of DNA by intercalating fluorochromes and thus cause a stoichiometric error in DNA content measurements [1, 6, 11, 12, 16, 26, 27]. The addition of antioxidants to the isolation buffer in many cases seems to have no measurable effect on reducing this interference ([7, 18, 27], this paper). Additionally, the quantity of inhibitor(s) is apparently environmentally regulated [28, 29], which makes this method unreliable even if the optimal antioxidant concentration for certain species appears to have been established. Therefore, using tissues that contain such compounds for flow cytometric estimation of DNA content should be avoided. In the present research, a test for the presence of staining inhibitors showed that while the leaves of all the studied species contained compounds that biased the fluorescence of the internal standard nuclei (basing on our experience, the shift of the G_0/G_1 peak of the internal standard by at least three channels on the 512-channel scale was considered as indicative of the presence of staining inhibitors in the cytosol of the cells of the target species), their seeds were free of them (Table 1). Nevertheless, to avoid conducting instrument calibration between analyses of different samples, the same buffers (containing PVP or PVP and β-mercaptoethanol, marked as A, B, or C) were used for both leaves and seeds. This was appropriate, because experiments using seeds of five species showed that there were no statistically significant differences between the 2C-values of their nuclei isolated with and without antioxidants in the buffer (Table 3, Figure 1). This conclusion is important, since in some laboratories buffers containing antioxidants are used for nuclei isolation even if the plant material does not contain secondary metabolites.

The histograms of the nuclei isolated from seeds were of better quality than those from leaves (Figures 1 and 2, Table 4). In some seed nuclei samples, especially in those containing PVP at high concentrations and β-mercaptoethanol in the buffer, an additional population of particles appeared (Figures 1(e) and 1(f), arrows); this was eliminated by gating and did not bias DNA content measurements. The signals probably corresponded to complexes of the PI with the antioxidant(s). For all the species but *P. padus*, the CV of the G_0/G_1 peak of the target species seed nuclei was statistically lower than in leaves (Table 4). Also the background debris level (expressed as DF) in most cases was lower when seeds were used for sample preparation instead of leaves (except *C. dammeri*, *C. divaricatus*, *C. veitchii*, and *P. padus*). In the leaves of some species, without the addition of antioxidants it was hard even to distinguish the peaks, and/or the peaks were shifted due to a decrease in fluorescence (Figure 2(a)). Even after supplementing the isolation buffer with antioxidants, the extent of debris in the leaf samples was obvious when compared to those from seeds; in some samples it was over 80% before gating was applied. The SSC and FSC showed the presence of additional particle populations in almost all samples of leaf nuclei (Figures 2(b) and 2(f), arrows), similar to the "tannic acid effect" [10]. They most probably resulted from aggregates of nuclei or parts of nuclei with unspecific particles and of diverse particles devoid of nuclei (inhibitors, antioxidants) with PI stain. We show that in the studied here species there is an association between the presence of inhibition effects and lower sample quality, even in cases in which DNA content measures do not differ. However, while seed histograms quality was higher than the one of leaf histograms in most species when using our protocols, in some cases it might be

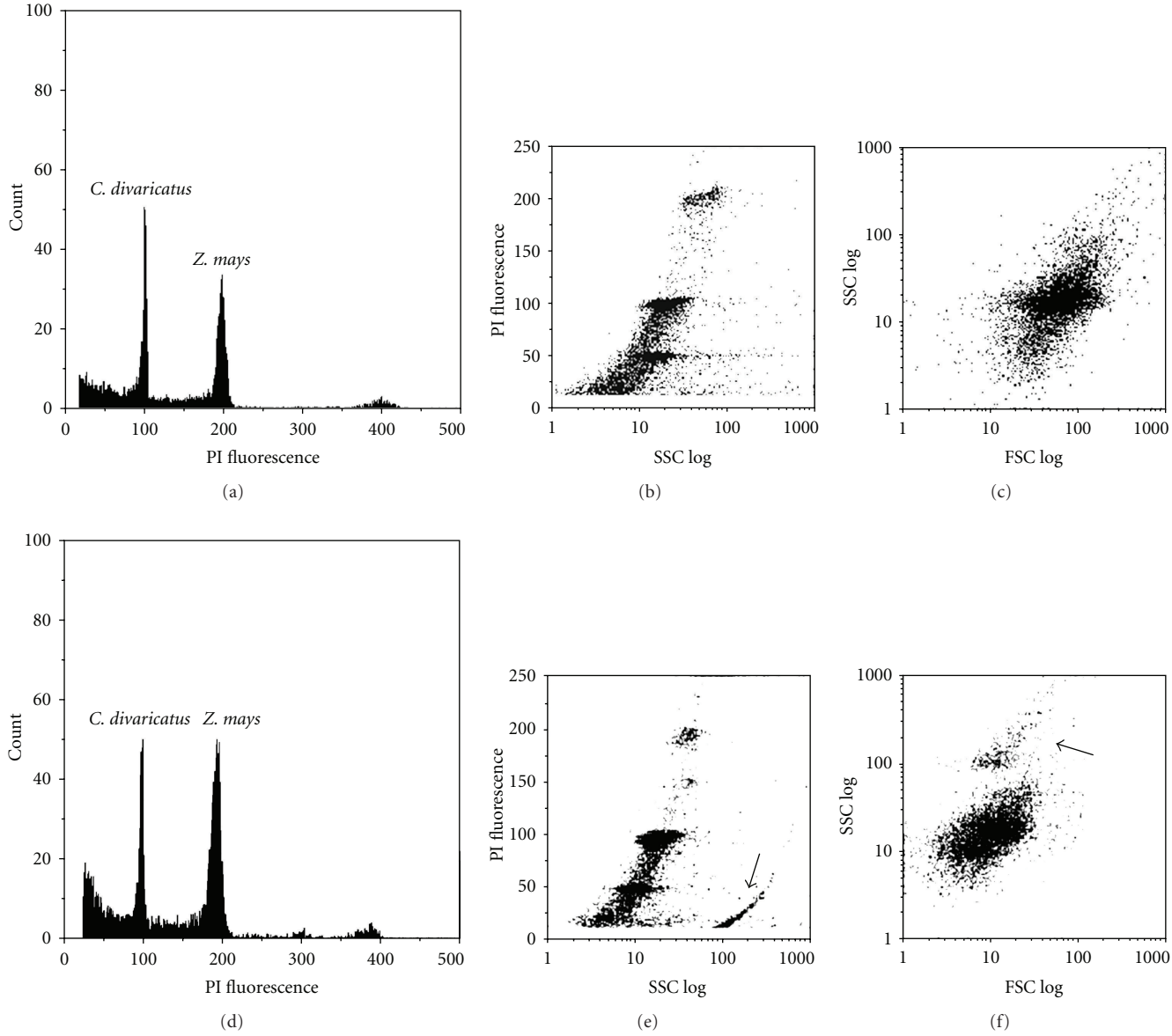

Figure 1: Histograms of PI fluorescence intensity (a, d) and dot plots on side scatter (SSC) versus PI fluorescence (b, e) and forward scatter (FSC) versus SSC (c, f) in the nuclei of seeds of *C. divaricatus* and *Z. mays* (internal standard) isolated using Galbraith's buffer (a–c) and Galbraith's buffer supplemented with 2% (w/v) PVP-10 and 15 mM β-mercaptoethanol. *C. divaricatus* does not possess the 4C nuclei that coincide with the 2C peak of *Z. mays*. Arrows indicate fluorescent particles without nuclei.

possible to achieve improved results from leaves using other refinements, notably other buffers and/or younger leaf tissue.

The 2C-values obtained for the leaves and seeds were not statistically different for seven out of 11 species (Table 2). However, in the leaves of *A. stolonifera* and three *Cotoneaster* species, the 2C-values were higher than in the seeds. A similar tendency was observed previously for *M. coronaria* [30] and *Eucalyptus globulus* [31]. However, in *Helianthus annuus* and *Brassica napus* a higher 2C-value was estimated in the seeds than those in the leaves [18]. Probably the material studied here contained interfering staining inhibitors that they could not be completely eliminated by addition of antioxidants, and thus the overestimated values were due to the reduction of the fluorescence of the internal standard nuclei. Another explanation of the differences between the 2C-values in leaves and seeds could be due to a different chromatin structure in those two organs. Differences in the chromatin condensation were previously observed in different tissues/organs of *Z. mays* [32, 33]. However, in the present experiments it is not likely that the lower estimation of DNA content in some seeds is due to differences in chromatin structure; such differences would be either present in or absent from all species and influence all the measurements, which was not the case. Also, taking into consideration that the DF was similar for both leaves and seeds in two out of four species for which the differences

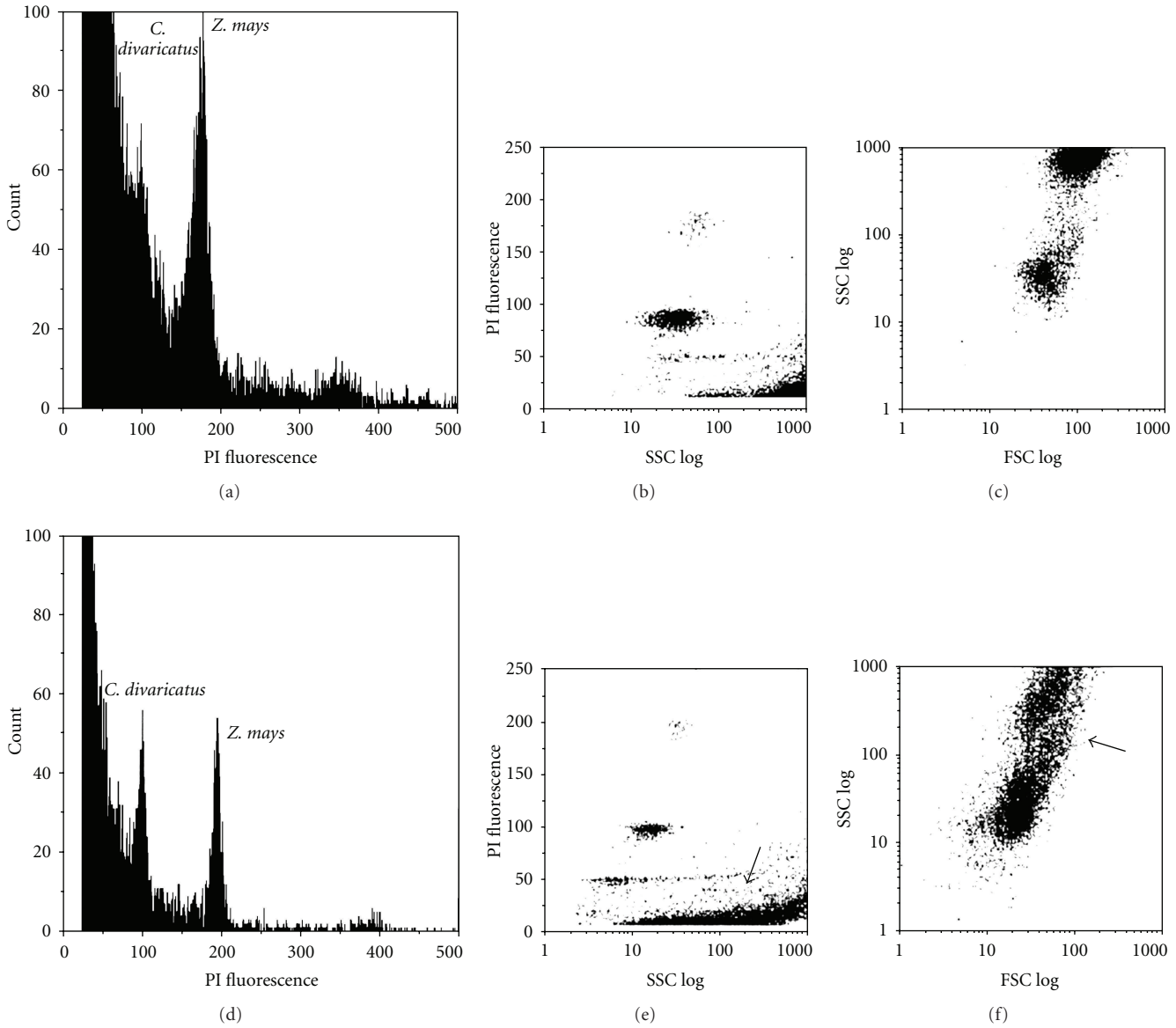

Figure 2: Histograms of PI fluorescence intensity (a, d) and dot plots on side scatter (SSC) versus PI fluorescence (b, e) and forward scatter (FSC) versus SSC (c, f) in nuclei of leaves of *C. divaricatus* and *Z. mays* (internal standard) isolated using Galbraith's buffer (a–c) and Galbraith's buffer supplemented with 2% (w/v) PVP-10 and 15 mM β-mercaptoethanol. *C. divaricatus* does not possess the 4C nuclei that coincide with the 2C peak of *Z. mays*. Arrows indicate additional populations of particles (see text for explanation).

in 2C-values occurred, it does not also seem that these were because of difficulties in measuring peak positions due to too much debris.

The 2C-values in the Rosaceae ranges from 0.20 to 7.30 pg [34]. Out of four subfamilies, a relatively large genome size occurs in the Spiraeoideae, which supports the polyploidy origin of this subfamily [1]. The genome size of the species studied here, most of which belong to Spiraeoideae, range from 1.15 pg/2C to 3.17 pg/2C. Based on the literature [1, 2, 14, 15, 30, 35], which provides 2C-values and/or ploidies for Spiraeoideae species related to those studied here, it can be assumed that they are mostly tetraploid, except for *Cotoneaster dammeri, M. floribunda,* and *P. elaeagrifolia*, which are diploid. Although cross-pollination between individuals with different ploidy levels is possible in the Rosaceae, no differences in ploidy were observed here between leaves and seeds originating from the same tree/shrub. Nevertheless, in seeds of species where there is a polyploidy series it is possible that hybridization between plants of different ploidies has occurred, and that the DNA content in the embryo may not correspond to that of the mother plant. In such cases, the leaves of the parent should be analyzed as a ploidy control.

Only for one species studied here, *M. floribunda* was the genome size previously established. Our values, 1.45 pg/2C in the leaf and 1.44 pg/2C in the seed (Table 2), are very close to

Table 4: Quality of the histograms of nuclei isolated from leaves and seeds of trees and shrubs belonging to the Rosaceae family. CV: coefficient of variation for the G_0/G_1 peak; DF: debris background factor.

Species name	CV of target species (%, mean ± SD)		CV of internal standard (%, mean ± SD)		DF (%, mean ± SD)	
	Leaves	Seeds	Leaves	Seeds	Leaves	Seeds
Amelanchier stolonifera	6.54 ± 0.52 a*	4.43 ± 0.43 b	4.09 ± 0.41 a	4.63 ± 0.31 b	57.8 ± 4.3 a	22.2 ± 3.6 b
Cotoneaster dammeri	5.73 ± 0.68 a	3.83 ± 0.39 b	2.82 ± 0.37^{ns}	3.12 ± 0.30	78.6 ± 8.7^{ns}	67.0 ± 18.5
Cotoneaster divaricatus	4.52 ± 0.21 a	3.35 ± 0.70 b	3.23 ± 0.51^{ns}	3.36 ± 0.45	83.1 ± 14.4^{ns}	78.0 ± 4.9
Cotoneaster hjelmqvistii	4.58 ± 0.54 a	4.04 ± 0.25 b	3.53 ± 0.41 a	3.98 ± 0.31 b	56.2 ± 10.3 a	38.8 ± 3.5 b
Cotoneaster horizontalis	5.66 ± 0.50 a	3.95 ± 0.40 b	4.17 ± 0.56 a	3.64 ± 0.33 b	73.9 ± 4.3 a	40.6 ± 2.8 b
Cotoneaster veitchii	4.52 ± 0.57 a	3.40 ± 0.76 b	3.25 ± 0.52^{ns}	3.51 ± 0.58	75.6 ± 13.2^{ns}	66.1 ± 22.4
Crataegus coccinea	5.68 ± 0.59 a	4.06 ± 0.35 b	4.46 ± 0.48 a	3.42 ± 0.52 b	47.1 ± 8.8 a	31.6 ± 8.5 b
Malus floribunda	5.84 ± 0.74 a	4.45 ± 0.25 b	3.78 ± 0.41^{ns}	3.79 ± 0.41	45.2 ± 6.3 a	30.1 ± 3.1 b
Prunus padus	5.94 ± 0.42^{ns}	5.95 ± 0.45	3.60 ± 0.39^{ns}	3.64 ± 0.40	52.8 ± 10.2^{ns}	53.9 ± 7.8
Pyrus elaeagrifolia	5.86 ± 0.71 a	4.82 ± 0.17 b	3.81 ± 0.36 a	3.36 ± 0.19 b	44.2 ± 6.9 a	25.5 ± 8.8 b
Sorbus intermedia	5.78 ± 0.42 a	4.36 ± 0.46 b	3.92 ± 0.30 a	4.28 ± 0.36 b	53.4 ± 9.26 a	34.2 ± 6.0 b

*Values for leaves and seeds of the certain species and for the certain parameter (in lines) followed by different letters are significantly different at $P = .05$ (Student's t-test); ns: no significant difference.

those reported by Tatum et al. (1.46 pg: [15]). Polyploidy in the *Crataegus* genus has been extensively studied [2, 13, 14]; *C. coccinea* is reported to be tetraploid, and the DNA content for 4*x Crataegus* ranges from 2.74 to 3.34 pg/2C. Estimations obtained here, 3.16 pg (for leaves) and 3.17 pg (for seeds), fall within this range. There are only single estimations of the genome sizes of species of the genera *Amelanchier, Cotoneaster, Pyrus,* and *Sorbus*, but not of those reported here [34]. Thus, our measurements provide 10 new entries to the plant C-value database.

Presently, seeds are not often used for genome size estimation; besides their many advantages they also have some disadvantages. They are convenient because they can be transported and stored dry with no distance or time limit and analyzed at a convenient time. For example, using seeds for genome size estimation has been proposed for desert plants, especially for those that do not develop leaves [20]. However, there can be some confusion in the interpretation of FCM results obtained from seeds due to the presence of cells with a higher than 2C DNA content [21, 22]. Nevertheless, if the whole seed is not suitable for use, it is usually possible to isolate a tissue or region in which most of the cells are arrested in the G_0/G_1 phase of the cell cycle (possessing 2C DNA). This may require a knowledge of seed biology and/or additional experiments to find the most suitable tissue for a particular seed type/species. If dissection of the radicle is needed, sample preparation involves additional time and precision; for small seeds, the use of a microscope may be required. Seeds are usually produced in abundance, although they may be rare or absent in some populations and years, and thus not available for DNA content measurement. Also, they have to be used with caution because in some families hybridization between species may occur, the Rosaceae being an example [30].

Within the Rosaceae family, seeds of *Cotoneaster* have been used previously for flow cytometric DNA content measurement for ploidy estimation, using external standardization and a two-step procedure, involving centrifugation [13, 14]. As shown here, this complex procedure can be successfully replaced by a simple and reliable one-step standard protocol, using the whole seed, the dissected radicle, or part of the seed containing the radicle. Such material is suitable for analysis without any other special pretreatment for sample preparation.

Our results demonstrate that the use of seeds may greatly simplify protocols in cases in which difficult leaf tissue requires special procedures. However, the simplification of protocols is one consideration among many. The final choice of tissue will depend on a variety of factors, including tissue availability, storage and transport concerns, the ease with which paternal DNA content can be inferred from that of progeny, requirements for sampling nonreproductive individuals, and the relative difficulty of seed and leaf preparation, both of which may vary considerably.

In conclusion, in the Rosaceae species containing staining inhibitors in the leaf cytosol, their seeds, which are free of such compounds, are a suitable alternative material for flow cytometric estimation of DNA content. The presence of staining inhibitors should be tested for each species, especially woody ones, regardless of the plant material used for measurement, and the composition of the nuclei-isolation buffer should be optimized for individual tissues/species. However, even after the addition of antioxidants, a stoichiometric error in the flow cytometric estimation of DNA content in "difficult" species can occur, and thus using plant parts that contain compounds which affect nuclei fluorescence should be avoided.

Abbreviations

FCM: Flow cytometry
PI: Propidium iodide
PVP: Polyvinylpyrrolidone
SSC: Side scatter
FSC: Forward scatter
DF: Debris background factor.

Acknowledgments

The authors thank Professor J. D. Bewley and P. Kron (University of Guelph, Canada) for critical comments on the manuscript.

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Hindawi Publishing Corporation
Journal of Botany
Volume 2010, Article ID 320609, 7 pages
doi:10.1155/2010/320609

Research Article

Improved and Reproducible Flow Cytometry Methodology for Nuclei Isolation from Single Root Meristem

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Received 29 January 2010; Revised 30 March 2010; Accepted 6 May 2010

Academic Editor: João Loureiro

Root meristems have increasingly been target of cell cycle studies by flow cytometric DNA content quantification. Moreover, roots can be an alternative source of nuclear suspension when leaves become unfeasible and for chromosome analysis and sorting. In the present paper, a protocol for intact nuclei isolation from a single root meristem was developed. This proceeding was based on excision of the meristematic region using a prototypical slide, followed by short enzymatic digestion and mechanical isolation of nuclei during homogenization with a hand mixer. Such parameters were optimized for reaching better results. Satisfactory nuclei amounts were extracted and analyzed by flow cytometry, producing histograms with reduced background noise and CVs between 3.2 and 4.1%. This improved and reproducible technique was shown to be rapid, inexpensive, and simple for nuclear extraction from a single root tip, and can be adapted for other plants and purposes.

1. Introduction

Meristematic root tissue has been the object of studies ranging from chromosome analyses to cell cycle and its physiological regulations [1]. These events comprise rates of proliferating and quiescent cells, characterization of cell subsets and elucidation cell cycle length and progression, besides effects of various putative modulators and inhibitors (e.g., hormones, growth factors, and toxins) and of environmental conditions on the cell cycle, which can be screened by flow cytometry—FCM [2].

In chromosome analyses by flow sorting [3–8], root tip meristems have proved to be advantageous suppliers of metaphase chromosomes for isolation, thanks to the great readiness of cells to synchronize. Unlike *in vitro* cultures, namely, cell suspensions and leaf protoplasts, root tips represent a cheaper, easier to handle and more karyologically stable experimental system [3, 6, 9].

Considering the radicular meristem cell cycle, FCM has been considered a fast and accurate method to evaluate nuclear DNA content. This procedure allows monitoring the production of high-quality commercial seed lots after priming treatments [10]. For instance, the effect of osmopriming in pepper and tomato has been followed by FCM, employing embryonic root tips as material source [11–16].

Root meristems have also been required in other FCM applications to substitute the frequently used leaves in current protocols [17, 18]. This preference for root material from some species is based on decreased contamination of samples with plastid DNA and absence of secondary metabolites or other disturbances, which significantly reduce DNA yield and quality [19] by affecting fluorochrome accessibility to DNA [17]. In addition, the use of root tips does not involve storage length problems, especially when material collection is carried out at long distances [17, 18, 20]. In order to prove the reliability of employing radicular material, some authors have compared radicle and leaf histograms of pepper [12] and tomato [13, 14], and corroborated their 2C DNA content.

However, meristematic root tips show low yield of material per tip [21]. To overcome this limitation, different FCM procedures have been described to supply suspensions with a

large quantity of nuclei or chromosomes, although requiring a number of root meristems per sample. Chiatante et al. [21] developed a protocol for nuclei isolation, denominated gentle hand-homogenization, using a glass rod and modified devices. Sgorbati et al. [22] also employed a glass rod for the same purpose, recurrent by Sgorbati et al. [23] and Lorbiecke and Sauter ([24], with modifications). A further adopted methodology [3, 26–30] refers to the chopping technique established by Galbraith et al. [25], and other researchers used a mechanical homogenizer [1, 5]. Table 1 informs about other references that show relevant data upon implementation of FCM procedures using roots.

Considering the increasing demand for the use of roots as material source in FCM or in general studies for DNA content quantification, cell cycle analyses, seed lot evaluation, cytogenetic study, comet assay applications, and the difficulty in obtaining adequate nuclei amounts from radicular material, we describe here an improved and reproducible protocol for nuclei isolation from a single root meristem, using *Allium cepa* as a model.

2. Material and Methods

2.1. Plant Material. The species *Allium cepa* L. cv. Alice, seeds of which were kindly supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic), was used as a model. The procedures and analyses were carried out at the Cytogenetic and Cytometry Laboratory, General Biology Department, UFV, Brazil.

2.2. Rooting and Fixation. Onion bulbs were placed in contact with water for root induction at room temperature. After reaching 1.5–2.0 cm of length, primary roots were fixed with a methanol (Merck, http://www.merck-chemicals.com/, product number 1060091000): acetic acid glacial (Merck, product number 1000631000) solution 3 : 1 (v/v) and stored at $-20^{\circ}C$ until processing for flow cytometric analysis.

2.3. Excision of the Meristematic Region. In order to obtain as much as possible of the meristematic region alone, the following strategy was adopted. Firstly, we have developed a homemade slide as helper for cutting out the root tips. On this prototype slide (Figure 1(a)), one onion root was placed in the longitudinal fissure and the meristem, visually identifiable by its whitish color, was transversely excised in its upper extremity (between the meristematic region and the elongation zone) and in its lower end (between the meristematic region and the root cap), using a brand new razor blade, under stereoscopic microscope. This simple device allowed immobilization of the root as well as perpendicular and precise cuts, in an uninterrupted course, at the meristem boundary.

2.4. Nuclei Extraction. To remove fixative solution, the meristems were washed in distilled water three times of 10 minutes each. Next, they were softened with a pectinase solution (Sigma, http://www.sigmaaldrich.com/sigma-aldrich/home.html, product number P2736) diluted in distilled water in proportion 1 : 10 (enzyme: water) at $33^{\circ}C$ for 30 minutes. The meristems were subsequently washed again.

A single meristem was carefully transferred to a 2-mL microtube (Axygen, http://www.axygen.com/, product number MCT-200, Figure 1(b)) containing 300 μL of 4′,6-diamidino-2-phenylindole (DAPI) buffer (Partec GmbH, http://www.partec.com/cms/front_content.php, product name CyStain UV Ploidy, code number 05-5004). Nuclei suspensions were obtained with use of a commercially available mini hand mixer (Figure 1(c)) by homogenizing the solution with 2, 4, 6, 8, or 10 pulses (one pulse corresponds to approximately one second), in vertical movements, being careful to avoid twisting movements. After homogenization, the mixer was washed with 300 μL of the same buffer. Nuclear suspensions were sieved through 30 μm mesh nylon filter (Partec GmbH, order number 04-0042-2316) into cytometer reading tubes (Partec GmbH, code number 04-2000). Additional 600 μL of the buffer were dropped to wash the 2-mL microtubes, and the suspension was then filtered again.

2.5. Flow Cytometric Analysis. After 15–20 minutes in the dark at room temperature, the nuclear suspensions were analyzed with a PAS-III flow cytometer (Partec GmbH, code number 15-01-1000), equipped with a UV lamp emitting at 378 nm, excitation filters (KG 1, BG 38 and UG 1), a GG 435 long-pass barrier filter for blue fluorescence, and a TK 420 nm dichroic mirror to provide epi-illumination. The equipment was carefully calibrated and aligned using microbeads and standard solutions according to the manufacturer's recommendations. FlowMax software (Partec GmbH) was used to process the data. The instrument gain was adjusted in such way that the G_1 peak was positioned on channel 200 of the histogram of relative DNA content.

Three repetitions were carried out for each homogenization test. Assuming the best results, based on lowest CVs, ten samples were processed and analyzed each day, for two days, totalizing twenty samples, and the experimental procedure was executed by different researchers.

2.6. Cytological Analysis. Through cytological analysis we evaluated the nuclear quality (in regard to shape, integrity and isolation) after extraction procedures with 2-, 4- and 6-pulse homogenization.

Nuclei suspensions were transferred to 2-mL microcentrifuge tubes (Eppendorf, http://www.eppendorf.com/int/?l=1&action=start, order number 022431048) and centrifuged at 100 $\times$ g for 5 minutes. After discarding the supernatant, the pellets were stored at $-20^{\circ}C$ for 15 minutes in 2 mL of methanol (Merck): acetic acid glacial (Merck) solution 3 : 1 (v/v). The fixed materials were centrifuged again and the pellets were resuspended in 100 μL of fixative solution by brief vortexing. Two drops of this nuclei suspension were dripped onto a very clean slide. The slide was immediately air-dried by fast wave movements, stained with a solution of 0.5 μg/mL DAPI in PBS buffer (pH 7.4), covered with a cover slip (25 $\times$ 25 mm, Corning,

Table 1: Data of nuclear FCM analysis from root meristem according to methodology employed.

Meristem source	Root tip length (mm)	Root tip/sample	CV (%)	Nuclei/sample	Species	References
Method-gentle hand-homogenization						
Root	0–2	100 (unclear)	4.4 and 5.8	5×10^6–1×10^7	*Pisum sativum*	[21]
Method-glass rod						
Root	0–2	10	2.6 (only control)	—	*Pisum sativum*	[23]
Root	—	—	—	$\geq 1 \times 10^4$	*Oryza sativa*	[24]
Method-chopping						
Radicle	1	5–10	—	821–3852	*Solanum lycopersicum*	[11]
Radicle	1	≥20	—	$\geq 1 \times 10^4$	*Capsicum annuum*	[12]
Radicle	—	5	—	—	*Solanum lycopersicum*	[13]
Radicle	—	70–80	—	$\sim 6 \times 10^3$	*Solanum lycopersicum*	[14]
Radicle	1	5	—	5×10^3–1×10^4	*Solanum lycopersicum*	[15]
Root	—	10–30	—	1×10^4 (unclear)	*Arabidopsis thaliana*	[26]
Root	—	—	—	—	*Solanum tuberosum*	[27]
Root	~ 2	9	—	8×10^3–1×10^4	*Allium cepa*	[28]
Root	2–3	20		8×10^3–1×10^4	*Secale cereale*	[29]
Root	2–3	20	—	8×10^3–1×10^4	*Triticum aestivum*	[29]
Root	2–3	7		8×10^3–1×10^4	*Vicia faba*	[29]
Root	~2	9	—	8×10^3–1×10^4	*Allium cepa*	[30]
Radicle	1	3–5	—	3469, 4425, 7547, 18874	various	[31]
Radicle	—	15	—	1×10^4	*Hordeum vulgare*	[32]
Method-mechanical homogenizer						
Root	1–2	—	—	—	Various	[1]
Root	1	1 (and >1)	—	1×10^5/mL	*Pisum sativum*	[5]
Root	0.5–1	50	—	$\sim 1.5 \times 10^5$	Various	[19]
Root	2	—	—	—	*Vicia faba*	[33]

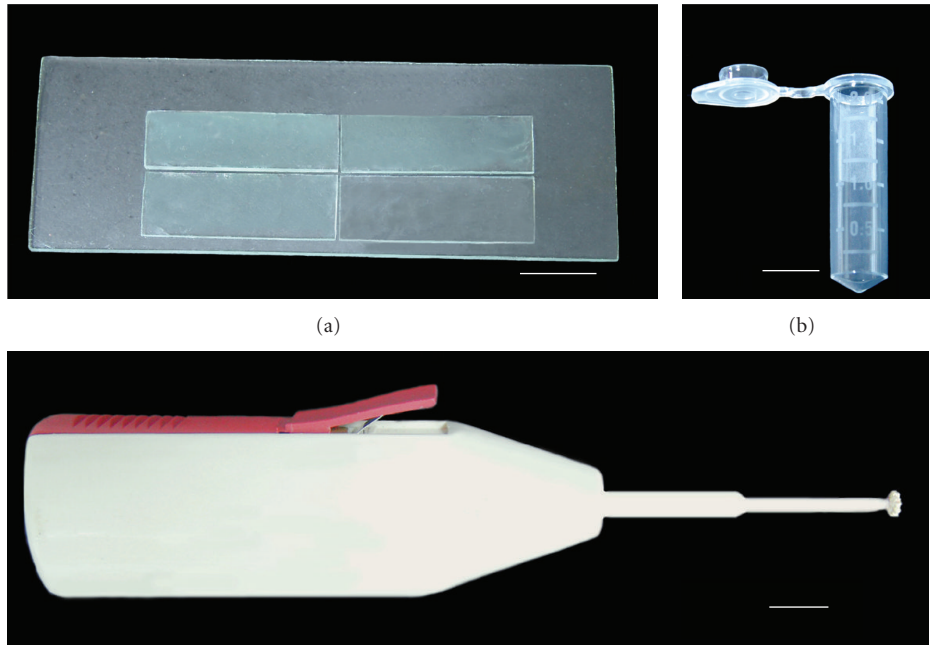
CV: coefficient of variation; —: data not shown.

http://www.corning.com/lifesciences/worldwide.aspx, product number 2865-25) and sealed with nail polish.

Images of DAPI-stained nuclei were captured with a DP71 video camera (Olympus, http://www.olympus-global.com/en/, product number D705-2), mounted on a BX-60 fluorescence microscope (Olympus), with a 100x objective lens and a WU filter (wide band cube filter: excitation wavelength 372 nm and emission wavelength 456 nm). The frame was digitized using the Image Pro-Plus 6.1 software (Media Cybernetics).

3. Results and Discussion

The use of a prototype slide (Figure 1(a)) facilitated the cutoff as much as possible of only the meristematic region of the onion root. We considered this strategy to be important for generating FCM suspensions free of nonmeristematic tissue, when the focus is the cell cycle study. This nonmeristematic tissue refers to the elongation zone, which is located just above the meristem region. Thus, the endoreduplicated cells commonly present in this zone cause the appearance of additional peak(s) on the histogram, whose interpretation can become more difficult. Some fine FCM works have mentioned a fixed length for sectioning the



(a) (b)

(c)

Figure 1: (a) Homemade prototype slide developed in our laboratory: on a brand new slide, four diamond-cut slide pieces were attached with commercial glass glue so that they could form two fissures—the longitudinal one, where a root is allocated, and the narrow transversal one, for the passage of a razor blade in a steady way. (b) Boil-proof 2-mL microtube (Axygen). (c) Mini AA battery operated hand mixer, commercially available and inexpensive (dimensions: $16 \times 3.5 \times 2.5$ cm). Bars = 1 cm.

root tip independently of the meristem size [21, 23, 29, 33]. Unfortunately, the practical manner how this was done has not been clearly described. Nevertheless, if the percentage of nuclei exclusively from meristem is considered critical, then the protocol should look into ways to delimit and extract this specific region. Such caution might be important for improving accuracy in flow cytometric analysis especially for very small material like root tips.

Preliminary tests were performed with 2-, 4-, 6-, 8-, and 10-pulse homogenization to select the best nuclei isolation, based on CVs of flow histograms as parameter of choice. The corresponding histograms are shown in Figures 2(a)–2(d), except for the 10-pulse homogenization test. The 6-pulse homogenization (Figure 2(c)) was adopted for *A. cepa*, which showed the lowest CV values. Histograms were generated from twenty individually processed roots, showing CVs that varied between 3.2 and 4.1%. The number of nuclei (G_0/G_1 peak) measured for each meristem ranged from 3108 to 7532. In fact, the histograms shown in Figure 2 only serve as a comparative guidance, since their high quality will depend on minor handling adjustments made by the executor (e.g., the way for homogenizing and pulse number) in accordance with the species studied, as happens in usual leaf chopping procedure in which some handiwork adjustments are made.

To certify nuclear quality, in regard to shape, integrity and isolation, the 2-, 4-, and 6-pulse homogenization tests were cytologically analyzed. The others (i.e., 8 and 10 pulses) were excluded due to inadequate histogram patterns, denoted by an excess of debris. In three trials, spherical shaped and intact nuclei were observed, characterizing preserved morphology. However, nuclei in clusters and surrounded by cytoplasmatic residues were formed in the first trial (2-pulse homogenization, Figure 3(a)), whereas in the second one (4-pulse) nuclei were isolated, but still evidenced cytoplasm (Figure 3(b)). As expected, the optimal 6-pulse test corroborated prior data (represented by the histogram in Figure 2(c)) inasmuch as completely extracted nuclei were visualized (Figure 3(c)).

Acid methanol fixation was considered an important step due to the promoting material storage until FCM running. Besides, this fixation process did not alter the nuclear suspensions quality. The use of this nonadditive fixative (i.e., which acts on the tissue without chemically changing it) does not interfere with quantitative staining involving DNA intercalators [20]. In the present paper, the flow cytometric protocol using DAPI (which preferentially binds to AT-rich regions) was not hampered by the fixation process.

Concerning the enzymatic digestion, this step was included to soften hard root tissue and consequently render a more gentle homogenization so as to avoid intense mechanical stress and, consequently, damaged nuclei and cellular debris. Short exposure to pectinase solution was not enough to degrade or modify neither nuclei morphology nor DNA structure. In the same way, Doležel et al. [3] discussed that longer enzymatic treatments may cause increased stickiness and even disintegration of chromosomes, in cases of flow sorting approaches.

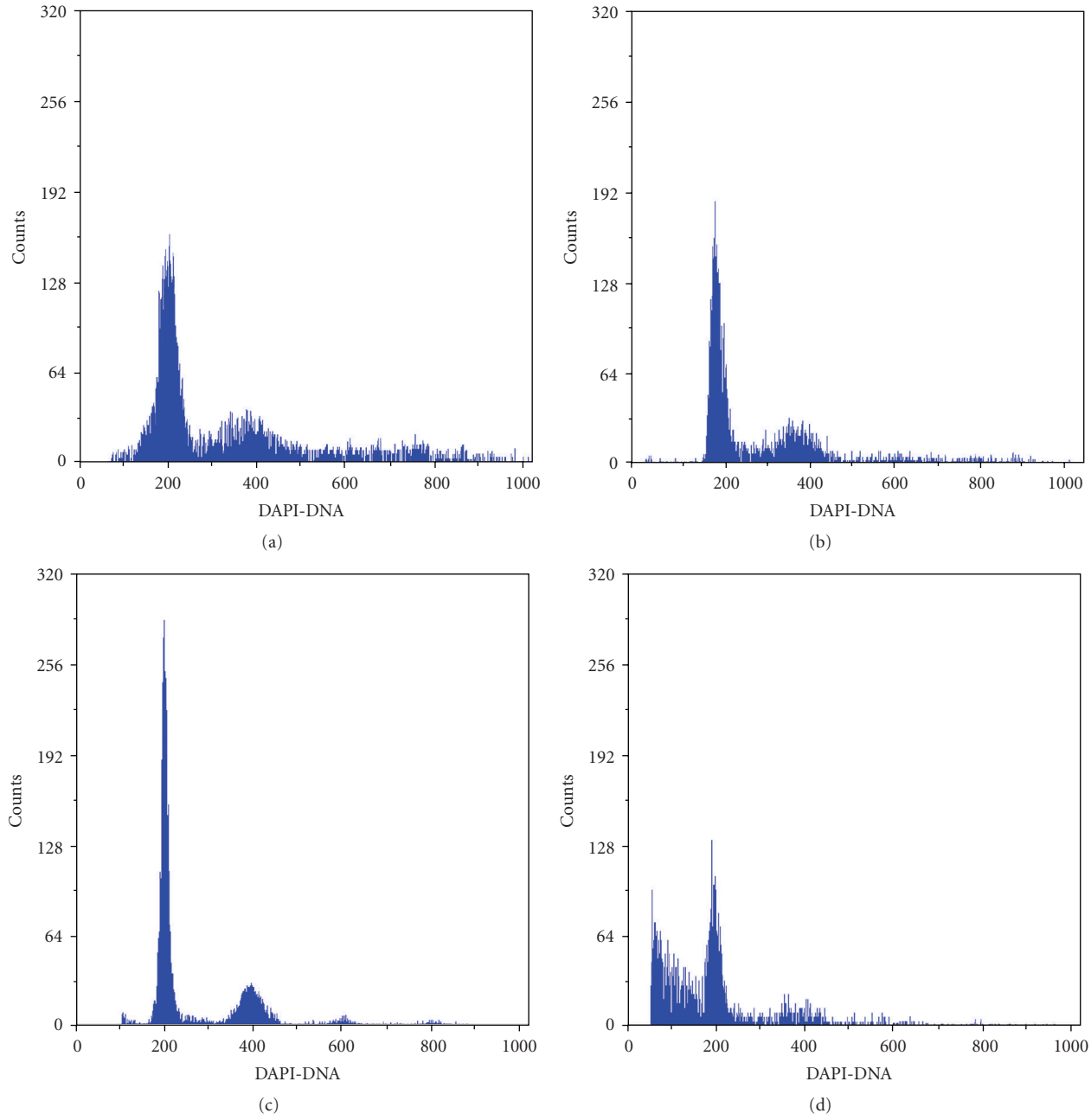
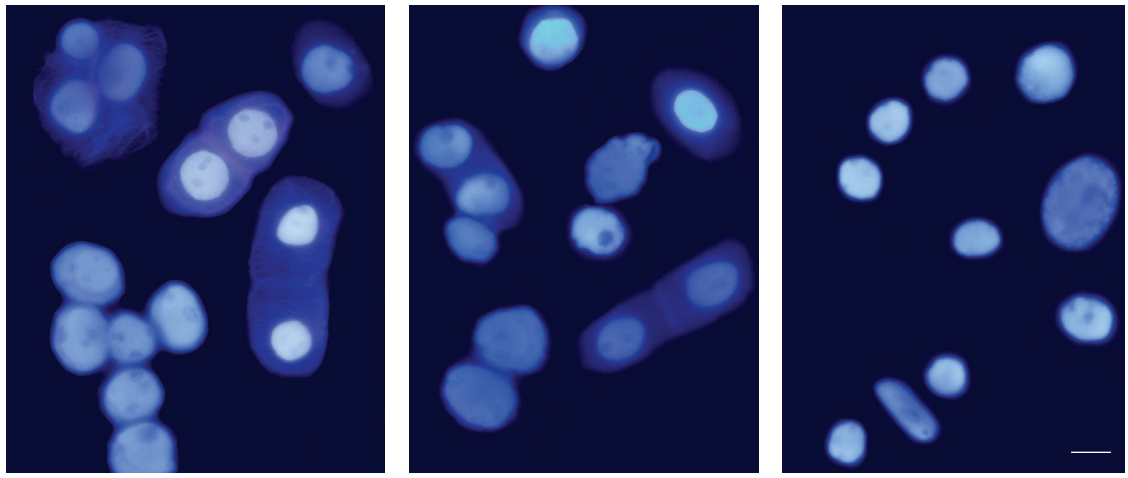


Figure 2: Representative flow histograms of DAPI-stained nuclei from *Allium cepa* root meristem after 2- (a), 4- (b), 6- (c), and 8- (d) pulse homogenization tests, showing in G_0/G_1 peaks: 4120 nuclei (CV 9.16%), 3855 nuclei (CV 7.36%), 4723 nuclei (CV 3.2%) and 2685 nuclei (CV 6.47%), respectively. Note that the most adequate histogram corresponds to 6-pulse homogenization (c).

In the present paper, use of the one-speed pulse mixer as well as the previous digestion step, facilitated the dissociation of one meristem at a time and consequently allowed the liberation of nuclei with minimal damages. Differently, Chiatante et al. [21] reported a failed attempt to obtain-high quality nuclear suspensions when they used a certain homogenizer. The mechanical stress caused by such instrument yielded nuclei with twisted shapes. But, other authors have applied the mechanical homogenizing method successfully, though processing more than one root tip per sample ([1, 5, 19]; see Table 1). Besides, many researchers have processed samples also with more than one root tip [11–15, 21, 23, 26, 29, 32] to compensate for the low quantity of isolated nuclei from the small-sized root meristem. However, the analysis of individual root meristem is fundamental in some studies, such as for investigating treatment effects or when plant number is limited.

The FCM results showed that the number of analyzed particles per root (range: 3108 to 7532) did not reach the quantity (10000) commonly found in leaf cytometric assays,



(a) (b) (c)

Figure 3: Morphological analysis of DAPI-stained nuclei, isolated from single onion root meristem after 2- (a), 4- (b) and 6- (c) pulse homogenizations. Note that after running of 6 pulses the nuclei were well preserved, isolated and showing no residual cytoplasm. Bar = 10 μm.

but they basically corresponded to DAPI-stained nuclei, as confirmed by the negligible background on the left of the G_0/G_1 peaks (Figure 2). Therefore, low CVs (below 5%), which are considered acceptable for FCM assessments [20], were achieved. These characteristics reiterate the great quality of histograms and, consequently, the high resolution of the proposed technique. Thus, it is important to emphasize these parameters in order to demonstrate the reliability of the cytometric procedure applied. Unfortunately, only few authors have regarded the inclusion of these data and CV values (Table 1), and among those, only Sgorbati et al. [23] assessed a CV value lower than the one found in this study. A possible explanation for the difficulty in obtaining low CVs from roots refers to the heterogeneity of cell cycle length in different tissue domains, belonging to derivative region immediately proximal to the apical meristem [1].

Some procedures from this new flow cytometric methodology, particularly digestion time and homogenization pulses, were specifically suitable for *A. cepa*. Nonetheless, these steps can be adjusted to other species. In our laboratory routine, results showing same quality have been obtained for other species, like eucalyptus, soybean and maize. Moreover, considering the applicability of this work for estimation of nuclear DNA content and cell cycle analyses in roots, additional aspects may also be adapted for other purposes, such as obtaining chromosome suspensions for flow sorting and radicle studies. Apart from use in FCM approaches, obtaining individualized nuclei suspensions could also be applied to the comet assay technique (single-cell gel electrophoresis), one of the most used assays for the assessment of DNA damage repair [34].

We conclude that our FCM protocol was reproducible and efficient to provide adequate nuclear suspensions for this approach from a single root meristem. Besides, the simplicity, rapidness and low cost of the described technique should not be overlooked.

Acknowledgments

The authors thank Conselho Nacional de Pesquisa (CNPq, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Brazil) for providing financial support for this paper.

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