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First genome size assessments for *Marshallia* and *Balduina* (Asteraceae, Helenieae) reveal significant cytotype diversity

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Abstract. The genus *Marshallia* is made up by seven to ten species of perennial herbs growing mainly in open habitats, whereas the genus *Balduina* is represented by three sympatric species; two perennial herbs and one annual, growing in open pine forest habitats. Both genera belong to the family Asteraceae, tribe Helenieae, and are endemic to the southeast United States, in North America. Cytogenetic studies concerning these two genera are scarce and genome size data is lacking for both. The main goals of this study were to (i) generate novel insights into the evolution of the genome size and (ii), contribute to filling existing gaps on our knowledge of the Asteraceae family from this point of view. Nuclear DNA contents range from 11.42 pg/2C in *Marshallia trinervia* to 31.58 pg/2C in *Marshallia mohrii*. The combination of genome size with chromosome data (and inferred cytotypes) suggests the existence of multiple cytotypes, and provides interesting insights into the potential impact of polyploidy in the evolution of these genera in general, and the shaping of genome size diversity, in particular.

Keywords: Barbara's buttons, chromosome counts, Compositae, nuclear DNA content, karyology, polyploidy.

INTRODUCTION

The genus *Marshallia* Schreb. (Asteraceae: Helenieae), commonly known as Barbara's buttons, is endemic to the southeast United States of America (Hansen and Goertzen 2014). This small genus is made up of seven (Baldwin 2009; Watson 2006) to ten species (Weakley 2020) of perennial herbs, which grow mainly in open habitats such as pine forests and roadsides, although

some species show preference for wet habitats as bogs, shoals or stream sides.

Morphologically, the genus is characterized by possessing discoid inflorescence heads of deeply lobed, rotate corollas that are colored either white or pink. Some of its morphological features are shared with other groups of Asteraceae (Baldwin 2009). This author placed the genus within subtribe Marshalliinae, closely related to Gaillardiiinae (which includes *Balduina* Nutt., *Gaillardia* Foug., and *Helenium* L.) in the tribe Helenieae, but its sister group has not yet been clearly established (Baldwin and Wessa 2000). Although species of *Marshallia* can be difficult to distinguish from each other based on morphological characters, a more recent study carried out by Hansen and Goertzen (2014) revealed that nuclear ribosomal ITS sequences serve as an acceptable DNA barcode marker in the genus, with sufficient nucleotide differences to discriminate amongst most species.

The genus *Balduina* Nutt. is endemic to the south-east United States, and it is represented by just three sympatric species, two perennial herbs and one annual (Keener 2006). Parker and Jones (1975) putatively related this genus to the tribe Helenieae based on an analysis of flavonoid and sesquiterpene lactone composition.

Genome size (GS, usually estimated as the 2C-value), refers to the total amount of DNA in an unreplicated somatic nucleus (i.e. holoploid genome size, Greilhuber et al. 2005). This parameter is considered as a biodiversity trait given the 2,400-fold variation encountered among land plants (Pellicer et al. 2018), with representatives having some of the largest eukaryotic genomes so far reported (c. 300 Gbp/2C) in contrasting lineages such as the monocots and pteridophytes (Hidalgo et al. 2017). Certainly, the relevance of this parameter in the evolution of plants is without doubt and further supported by the multiple correlations reported between GS and several ecological, life cycle and karyological attributes (e.g. Bennett and Leitch 2005; Beaulieu et al. 2008; Knight and Ackerly 2002; Pellicer et al. 2010a; Pustahija et al. 2013; Pellicer et al. 2014).

Genome size diversity and evolution studies in the Asteraceae have been examined by several authors (e.g. Vallès et al. 2013, Vitales et al. 2019). However, achieving a comprehensive understanding of GS evolution in a family as large as the Asteraceae (c. 25,000 species) is challenging. In fact, only about 6% of the extant taxonomic diversity at the species level in this family has been studied from this point of view (Vitales et al. 2019). Despite the gaps in our knowledge, those studies have evidenced a relative high diversity of GS across species, ranging about 139-fold, mostly driven by the ubiquitous nature of polyploidy across the family. Indeed, the lack

of correlation between GS and chromosome number among diploids suggests that chromosomal rearrangements have a relatively minor impact on the overall DNA content at the family level (Vitales et al. 2019).

Although some species of *Marshallia* have recently been the subject of studies of nuclear gene regulation in non-model systems (Melton et al. 2019), and also of conservation biology (Knapp et al. 2020), cytogenetic studies concerning *Marshallia* or *Balduina* are very scarce and mostly restricted to chromosome counts. So far GS data are entirely absent for both genera according to the Plant C-values Database (<https://cvalues.science.kew.org>, Pellicer and Leitch 2020) as well as the family-specific Asteraceae Genome size database (<https://www.asteraceae-genomesize.com>, Vitales et al. 2019). For that reason, the main goal of this study was to provide new GS and chromosome data for most species of these genera, aiming at (i) generating novel insights into the evolution of this parameter and (ii) contributing to filling existing gaps on our knowledge of Asteraceae genome size evolution.

MATERIALS AND METHODS

Plant material

The species and populations studied as well as their origin and herbarium vouchers (deposited in the John D. Freeman Herbarium (AUA), of the Auburn University Museum of Natural History, Auburn, Alabama, USA) are shown in Table 1.

Nuclear DNA content assessments

Genome sizes of the target species were estimated using flow cytometry. *Pisum sativum* L. 'Express Long' (2C = 8.37 pg, Marie and Brown 1993) was used as an internal standard. Young, healthy leaf tissue (about 25 mm²) from each species was placed in a plastic Petri dish and chopped in 1,200 µl of LB01 lysis buffer (Doležel et al. 1989) with a razor blade. The suspension of nuclei was filtered through a nylon mesh with a pore size of 70 µm and stained for 20 min with 36 µl of propidium iodide (60 µg/mL; Sigma-Aldrich Química) and supplemented with 100 µg/ml ribonuclease A (Boehringer). For each individual, two replicates were prepared and processed on the flow cytometer. Measurements were made at the Centres Científics i Tecnològics de la Universitat de Barcelona using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Fla.). The instrument was set up with the standard configuration: excitation of

Table 1. *Marshallia* and *Balduina* species studied including population code and origin.

Species	Code	Voucher (in herbarium AUA)
<i>Balduina uniflora</i> Nutt.	B1	Live material from AU Davis Arboretum
<i>Marshallia caespitosa</i> Nutt. ex DC. var. <i>Caespitosa</i>	M26	Watson 12-01, Pottawatomie Co., OK
<i>M. graminifolia</i> (Walt.) Small	M1	Hansen 4951, Covington Co., AL
<i>M. graminifolia</i> (Walt.) Small	M39	Hansen 5814, Jackson Co., MS
<i>M. graminifolia</i> (Walt.) Small	M40	Hansen 5814, Beauregard Par., LA
<i>M. mohrii</i> Baedle and F.E.Boynton	M20	Hansen 5055, Bibb Co., AL
<i>M. mohrii</i> Baedle and F.E.Boynton	M21	Hansen 5056, Bibb Co., AL
<i>M. obovata</i> (Walt.) Baedle and F.E.Boynton	M3	Hansen 4956, Macon Co., AL
<i>M. obovata</i> (Walt.) Beadle and F.E.Boynton	M22	Hansen 5471, Macon Co., AL
<i>M. obovata</i> (Walt.) Beadle and F.E.Boynton	M34	Hansen 5786, Bullock Co., AL
<i>M. ramosa</i> Beadle and F.E.Boynton	M19	Hansen 5054, Ben Hill Co., GA
<i>M. ramosa</i> Beadle and F.E.Boynton	M38	Hansen 5795, Washington Co., FL
<i>M. trinervia</i> (Walt.) Trel.	M2	Hansen 4954, Lee Co., AL

the sample was done using a standard 488 nm air-cooled argon-ion laser at 15 mW power. Acquisition was automatically stopped at 8,000 nuclei. The half-peak coefficient of variation was calculated for both target plant material and the internal standard.

Chromosome counts

Root-tip meristems were obtained from achenes germinated on wet filter paper in Petri dishes at room temperature. Seedlings were pretreated with 0.05% aqueous colchicine at room temperature for 2.5 h. Material was fixed in absolute ethanol and glacial acetic acid (3:1) for 2 h at room temperature and stored in the fixative at 4°C. Samples were hydrolyzed in 1 N HCl for 5 min at 60°C, stained with 1% aqueous aceto-orcein for 4h, and squashed on slides in 45% acetic acid-glycerol (9:1). The best metaphase plates were photographed with a digital camera (AxioCam MRc5 Zeiss) mounted on a Zeiss Axio-plan microscope, and images were analyzed with Axio Vision Ac software version 4.2.

Phylogenetic tree and data mapping

In order to plot and visualize GS data from a phylogenetic perspective, GenBank ITS sequences from Hansen and Goertzen (2014) and an outgroup (*Helianthus annuus* L.) were downloaded using Geneious Prime 2020.1.2 (<https://www.geneious.com>), and aligned with CLUSTAL Omega (Sievers et al. 2011). A Maximum Likelihood tree was then constructed using the default settings and 10,000 bootstrap, as implemented in Geneious. Genome size data were plotted on the tree

using the *plotTree.wBars* function implemented in *Phytools* package (Revell 2012), and C-value scatterplots were carried out using *ggplot2* package (Wickham 2016), both available in R (R core Team 2019).

RESULTS

The results obtained for GS, complemented with chromosome numbers in some of the accessions are shown in Table 2. Illustrative chromosome pictures and the distribution of GSs from a phylogenetic perspective in *Marshallia* are depicted in Figure 1. In all investigated accessions, flow histograms with coefficients of variation below 3.5 were obtained, illustrating the high quality of the results obtained. As highlighted above, these two genera have never been studied from this perspective, and therefore, our results represent the first estimates for all of these species.

DISCUSSION

The combination of GSs with actual chromosome data (plus inferred cytotypes) provides interesting insights into the potential impact of polyploidy in the evolution of both *Marshallia* and *Balduina*. Semple and Watanabe (2009) attributed to the tribe Helenieae s. str., to which the two genera considered here belong, a secondarily derived base number of $x = 19$. However, all counts reported here as well as those previously recorded in the literature (see below) correspond to a primary base number of $x = 9$, one of the most frequent in the family Asteraceae.

Table 2. *Marshallia* and *Balduina* species studied including genome size measurements and chromosome counts.

Species	Code	N ¹	2C (pg)	2C (Mbp) ²	1Cx (pg)	HPCV plant	HPCV standard	2n	2n ³
<i>Balduina uniflora</i> Nutt.		2	12.96±0.00	12675	6.48	2.44±0.37	3.03±0.16	18*	72
<i>M. caespitosa</i> Nutt. ex DC.	M26	1	22.83	22328	5.70	1.87	2.85	36*	18,36
<i>M. graminifolia</i> (Walt.) Small	M1	1	12.74	12460	6.37	2.50±0.19	2.82±0.05	18	18
<i>M. graminifolia</i> (Walt.) Small	M39	1	12.72	12440	6.36	3.23±0.32	3.81±0.06	18*	18
<i>M. graminifolia</i> (Walt.) Small	M40	5	12.89±0.27	12606	6.45	2.42±0.48	3.01±0.37	18*	18
<i>M. mohrii</i> Baedle and F.E.Boynton	M20	1	16.70	16333	5.56	0.67±0.02	3.31±0.10	27*	36
<i>M. mohrii</i> Baedle and F.E.Boynton	M21	4	31.58±0.97	30885	5.26	1.27±0.95	3.08±0.28	54	36
<i>M. obovata</i> (Walt.) Baedle and F.E.Boynton	M3	3	13.60±0.51	13300	6.80	2.39±0.35	2.75±0.22	18	18
<i>M. obovata</i> (Walt.) Beadle and F.E.Boynton	M22	1	13.73	13428	6.86	2.95±1.60	3.71±0.07	18	18
<i>M. obovata</i> (Walt.) Beadle and F.E.Boynton	M34	1	13.92	13614	6.96	2.07±0.06	2.39±0.26	18*	18
<i>M. ramosa</i> Beadle and F.E.Boynton	M19	1	16.77	16401	5.59	1.25±0.32	2.20±0.13	27*	18
<i>M. ramosa</i> Beadle and F.E.Boynton	M19	2	23.92±0.12	23394	5.98	2.51±0.41	3.27±0.90	36*	18
<i>M. ramosa</i> Beadle and F.E.Boynton	M38	2	13.37±0.19	13076	6,68	3.02±0.29	3.44±0.25	18*	18
<i>M. trinervia</i> (Walt.) Trel.	M2	3	11.42±0.06	11169	5.71	3.21±0.26	3.30±0.26	18*	18

¹ N = number of individuals. ² 1 pg = 978 Mbp (Doležel et al. 2003). ³ Chromosome Counts Database (Rice et al. 2015). * Chromosome numbers inferred from nuclear DNA contents.

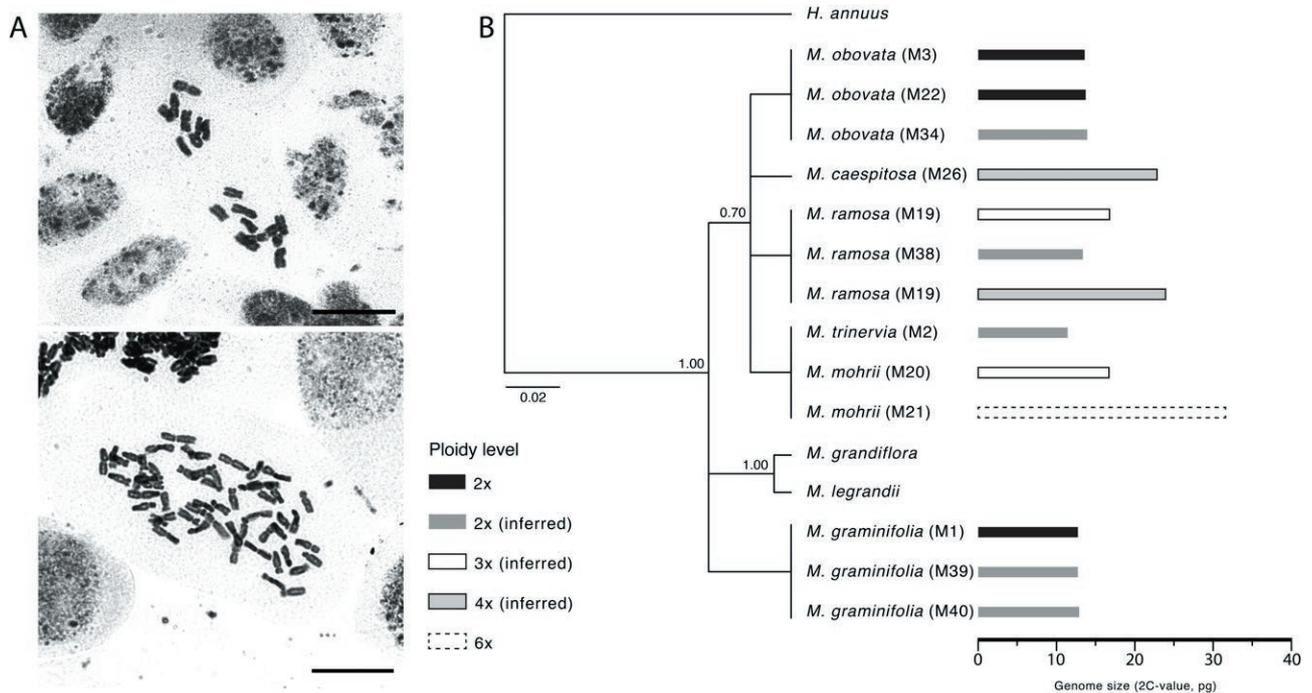


Figure 1. A. Illustrative chromosome counts in *Marshallia*: (top) *Marshallia obovata* (Walt.) Baedle and F.E. Boynton (M3, 2n = 18). (bottom) *Marshallia mohrii* (M21, 2n = 56). Scale bars are 10 µm. B. Phylogenetic mapping of genome size data (2C-values) based on ITS sequences from Hansen and Goertzen (2014). Inferred ploidy levels based on nuclear DNA contents are indicated.

Genome size and chromosome diversity in *Marshallia*

Nuclear DNA contents varied 2.76-fold in *Marshallia*, ranging from 11.42 pg/2C in *Marshallia trinervia* (Walt.) Trel. to 31.58 pg/2C in the population M21 of

Marshallia mohrii Beadle and F.E. Boynton (see Table 2). The large GS found in the latter, is further supported by the fact that this particular accession is a hexaploid, as confirmed by our chromosome counts (2n = 56, Figure 1a). Furthermore, a likely hybrid origin of this species,

with subsequent introgression has been suggested in the past. For example, Watson et al. (1991) and more recently Hansen and Goertzen (2014) suggested an allopolyploid origin of this species, hypothesizing that *M. trinervia* ($2n = 18$) could be one of the parents, which is supported by the very close phylogenetic relationship among both species (Hansen and Goertzen 2014). Other species possibly involved in the origin of *M. mohrii* could be either *M. caespitosa* or *M. ramosa*, given their relatively close phylogenetic relationship with this species (Hansen and Goertzen 2014). Considering the Federally Endangered status of this imperiled species, further research into its apparent cytotype diversity is warranted.

Of the two investigated accessions of *M. mohrii*, the specimen belonging to population M20 had a GS of 16.70 pg/2C. Compared to other confirmed diploid accessions in this study, such as *M. obovata* ($2C = 12.89$ pg) or *M. graminifolia* ($2C = 13.60$ pg), its nuclear DNA content is larger than would have been expected for a diploid accession. Several mechanisms could be invoked to provide an explanation for this GS, such as activation of amplification of repetitive DNA and/or polyploidy. Based on the value obtained for the hexaploid population of this species (M21, 31.58 pg/2C), a triploid cytotype could have, in theory, a similar GS as that found in population M20 (as inferred in Figure 1). However, to avoid excessive speculation, further studies would be needed to confirm this point including an actual chromosome count, and thus discard the existence of bursts of DNA amplification as the main driver of such genomic expansion. Concerning *M. caespitosa*, only one individual was analyzed in the present study (22.83 pg/2C, Table 2). From this value, a tetraploid cytotype can be also inferred (Figure 1), if compared with the results in chromosomally-confirmed diploid taxa. Certainly, both diploid ($2n = 18$) and tetraploid ($2n = 36$) levels are known in the species (Watson and Estes 1990), which makes our inference more feasible. *Marshallia ramosa*, the other possible genome donor of *M. mohrii*, is highly variable in morphology in the field, and also in GS (Table 2). In the present study, observed nuclear DNA content is compatible with three ploidy levels (2x, 3x and 4x; Figure 1), although only $2n = 18$ has been previously reported for this species (Watson and Estes 1990). Our results thus support a scenario where hybridization and introgression might have taken place, influencing changes in GS through the likely existence of multiple ploidy levels.

In contrast to the above-mentioned cytogenetic variability, data from *M. graminifolia* and *M. obovata*, suggest overall intraspecific GS stability, with values ranging only 1.02 and 1.01-fold among accessions, respectively. Our results confirmed that both species are diploid

(Table 2, Figure 1), as previously reported by Watson and Estes (1990), and therefore the small intraspecific differences in GS among them could have arisen through chromosomal reorganizations, as previously found in other Asteraceae (e.g. *Anacyclus*; Vitales et al. 2020).

Is genome size diversity mostly driven by polyploidy in Marshallia?

The nuclear DNA content estimates and somatic chromosome numbers from this study set up a scenario where polyploidy has played a significant and ongoing role in the evolution of *Marshallia*, influencing GS in particular. Genome sizes of around 12-13 pg/2C for the diploid level (i.e. $2n = 18$), 23-24 pg/2C for the tetraploid (putatively corresponding to $2n = 36$), and 31-32 for the hexaploid level (corresponding to $2n = 54$) were confidently inferred (Figure 1). In addition, two populations presented nuclear DNA amounts around 16-17 pg/2C, suggesting the existence of triploid representatives in the genus. If our overall ploidy inferences hold true, this would indicate that while *M. obovata* and *M. graminifolia* clades are essentially diploid, the clade including *M. mohrii* (3x and 6x), *M. ramosa* (2x, 3x, 4x) and *M. caespitosa* (4x) is cytogenetically highly diverse in a somewhat lineage-specific manner (Figure 1, Hansen and Goertzen 2014).

Polyploidy and whole genome duplications have been shown to have a direct impact on the GS, especially since it involves, at least, a duplication of the overall DNA content (Pellicer et al. 2018). However, genomic restructuring after polyploid formation can result in elimination of specific DNA sequences, leading to a loss of linearity in the accumulation of DNA, the so-called genome downsizing (Leitch and Bennet 2004). This phenomenon can be seen in *Marshallia*, where a reduction of the holoploid nuclear DNA content with increasing ploidy levels was observed, which was more patent at higher ploidy levels (Figure 2a). For example, bearing in mind that 2C-values of about 12-13 pg were found in diploid accession, nuclear DNA contents of ca. 18-20 pg would be expected in 3x, 24-26 pg in 4x, and 36-40 pg (6x) would be expected under the assumption of proportional genome expansion. However, the observed results are lower in each case (Table 2, Figure 2a). The impact of such reduction in *Marshallia*, is further illustrated by the fact that monoploid C-values (i.e. 1Cx) are lower in polyploids with respect to their diploid counterparts (Figure 2b).

As stated, genome downsizing in polyploids is a very common phenomenon in plants, and the Asteraceae family is no exception. Among other mechanisms, chromosome rearrangements after polyploidy, particularly

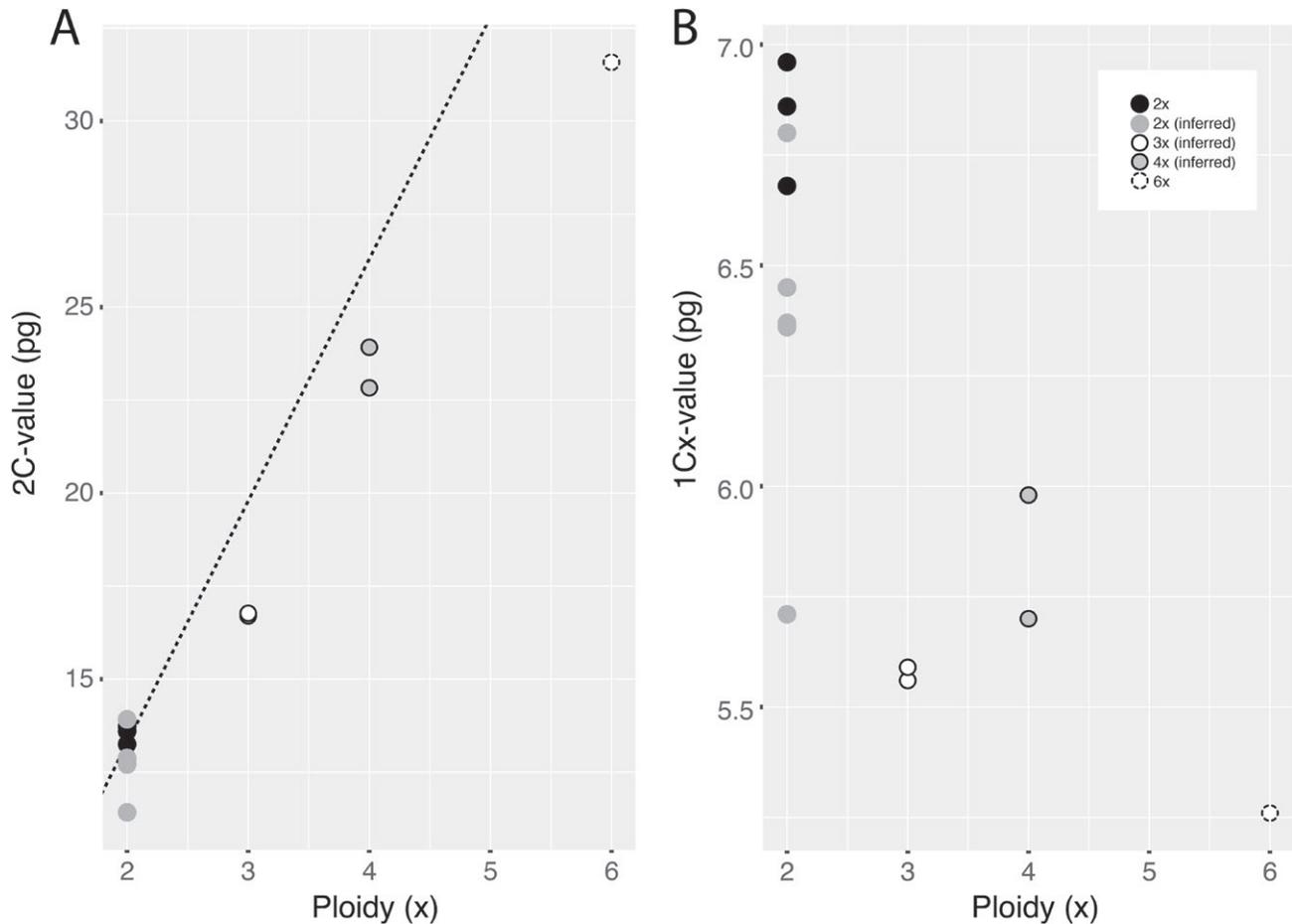


Figure 2. A. Scatter plot of observed 2C-values in *Marshallia* grouped by ploidy level. The dotted line represents the projection of expected 2C-values given a proportional increase of GS with ascending ploidy levels (note that the prediction is based on average 2C-values of diploid taxa). B. Scatter plot of observed 1Cx-values in *Marshallia* grouped by ploidy level, which illustrate the reduction of the monoplloid genome in ascending ploidy levels.

for relatively old genome duplication events, can influence this process (e.g. Leitch and Bennet 2004, Pellicer et al. 2010b, and references therein). In the genus *Artemisia*, even between closely related species, contrasting GS dynamics have been reported (Pellicer et al. 2013), involving changes in the number and distribution of repetitive DNAs, such as ribosomal DNA loci, which could influence changes in GS. However, in some other cases, genome size additivity has been also described, suggesting a more recent origin of such polyploids (e.g. Pellicer et al. 2010a). In other groups, both GS increases and decreases have been observed (e.g. *Nicotiana*, Leitch et al. 2008). A similar scenario was reported in the genera *Hieracium* and *Centaurea* (Bancheva and Greilhuber, 2006; Chrtek et al. 2009), where multiploid taxa revealed both genome downsizing or upsizing in each genus. The mechanisms underpinning changes on GS in polyploids

yet remain to be fully understood, but autopolyploidy and introgression could play a relevant role in determining the GS of the resulting polyploid.

Genus Balduina: nuclear DNA content evidences a potential unknown diploid cytotype

Available chromosome numbers compiled in the CCDB (Rice et al. 2015) for the genus indicate the presence of tetraploids in the species *Balduina atropurpurea* Harper. and *Balduina angustifolia* (Pursh) Robinson ($2n = 4x$; Parker and Jones 1975), and octoploids in *Balduina uniflora* Nutt. ($2n = 8x = 72$). Unfortunately, for our accession of *B. uniflora* we have only been able to estimate the GS and an actual chromosome count is thus, missing. Certainly, its nuclear DNA content falls within the range of GS for diploids encountered in the closely

related genus *Marshallia* (Table 2), suggesting that this accession could likely represent a diploid population. If this assumption holds true, then this finding would represent a new ploidy level report in the species, meaning a baseline level for chromosome evolution of the genus, which subsequently underwent several rounds of polyploidy. In any case, further chromosome research will be necessary to confirm this point and discard any other potential taxonomic issues.

CONCLUSIONS

We have performed the first GS assessments in the genera *Marshallia* and *Balduina*, complemented with chromosome counts and chromosome number inferences based on nuclear DNA content. The significant, ongoing role of polyploidy and hybridization in these genera has been discussed. In order to confirm some patterns deduced from the data, further research focused on chromosome counts should be carried out in all species lacking this information, complemented with GS in the remaining species of both genera. In *Balduina angustifolia*, the only annual species in the genus (Keener 2006), this research could be particularly interesting to test whether it shows a reduced GS associated with the faster life cycle than in perennials, as reported in many annual taxa (Pellicer et al. 2014, and references therein).

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