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Research Report

Contribution to the knowledge of genome size evolution in edible blueberries (genus *Vaccinium*)

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Abstract.
BACKGROUND: *Vaccinium* is one of the largest genera (*ca.* 500 species) of Ericaceae, well known for its edible and ornamental uses. Although there is certain karyological knowledge, information about genome size (GS) is scarce in the genus.
OBJECTIVE: The main goal is providing GS data for several *Vaccinium* species with prevalence in Europe and Western Asia and analysing global GS variation in the genus, considering available data and phylogenetic context.
METHODS: New GS assessments were obtained by flow cytometry and chromosome counts were verified. Phylogenetic analyses (using nuclear ITS, and chloroplastic *mat*K and *ndh*F) were performed by Bayesian inference and reconstruction of ancestral GS by maximum parsimony.
RESULTS: We obtained GS data for five *Vaccinium* species (13 populations). Three species are reported for the first time. Values (2C) ranged between 1.16–1.47 pg at the diploid (2n = 24) and between 3.13–3.16 pg at the tetraploid (2n = 48) levels. The five species here investigated have been placed and analysed in a reconstructed phylogenetic background (including 68 taxa).
CONCLUSIONS: GS values of *Vaccinium* can be considered "very small". The preliminary reconstruction of ancestral GS would point to a reduction in *Vaccinium*, although more data is needed to establish global GS evolutionary trend in the genus.

Keywords: Berries, C-value, chromosome counts, flow cytometry, nuclear DNA amount, nuclear DNA content

1. Introduction

Vaccinium L. is one of the major genera in family Ericaceae, with *ca.* 500 described species [1] although there are only 223 accepted species names (The Plant List, accessed 31st May 2019). All species are perennial, exhibit

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both self and cross-pollination and they have in common a small and pulpy berry as a fruit which in many cases is edible [2]. Most *Vaccinium* prefer cool regions of the Northern hemisphere, being broad in Europe, Asia and North America and absent in New Zealand, Australia and most of Africa; yet some species are also present in tropical regions such as Madagascar or Hawaii [3]. Fruits from several species of genus *Vaccinium*, commonly known as blueberries, enter into the category of the so-called berry fruits, which represent an increasingly consumed crop all over the world. Reasons for this recent boost of interest are the numerous health properties that are related to them. These berries are rich in anti-oxidant compounds, can alter the lipid metabolism, being beneficial for dietary purposes, and are rich in potassium and fibre [4]. They also have beneficial effects on cardiovascular and urinary diseases, and can improve brain function and cognitive ability [5, 6]. Besides, many *Vaccinium* have ornamental value [7].

The major type of cultivated blueberry (varieties of *Vaccinium corymbosum*) was developed in the last century [8] and several other *Vaccinium* have only recently been domesticated [9–11], in which interspecific hybridization has been an effective breeding strategy [12]. There are conservation projects involving wild *Vaccinium* to preserve traits potentially useful for breeding purposes, such as disease resistance, winter hardiness, low chilling requirement, adaptation to high pH soils, early ripening, late bloom or cold hardiness among others [8, 13]. However, there are still many knowledge gaps regarding the evolutionary and systematic diversity in this genus, which is essential information for conservation programmes as well as the development of new cultivars [e.g. 14, 15]. Molecular phylogenetic analyses have revealed that *Vaccinium* constitutes a polyphyletic group, with other closely related genera from tribe Vaccinieae (e.g. *Agapetes*, *Cavendishia, Paphia, Notoptora*) embedded within *Vaccinium* [3, 16]. In addition, the relationships among the major clades of blueberries have not been well resolved in previous phylogenetic treatments of the genus. One possible cause is the extent of hybridization, already noted as common in early cytological investigations [17]. This has led authors to advocate for the recognition of several clades or smaller groups rather than a large *Vaccinium* redundant to tribe Vaccinieae [3] and to propose sinking *Agapetes* in *Vaccinium* [18].

From the karyological point of view, *Vaccinium* also shows a certain level of evolutionary complexity. Species of this genus have many ploidy levels derived from a single base chromosome number, $x = 12$, ranging in nature from the diploid to the hexaploid. Auto- and allopolyploids are also common. Interspecific triploids, pentaploids, octoploids and nonaploids have been obtained for domestication purposes [19]. The most extensively studied species of *Vaccinium* are those from section *Cyanococcus* (including different ploidy levels) and section *Oxycoccus* (only diploids) [20]. Other sections such as *Myrtillus* and *Vitis-idaea* (only diploids), section *Hemimyrtillus,* including *V. arctostaphylos* (only tetraploids), and section *Vaccinium*, including *V. uliginosum* (different ploidy levels) have also been largely investigated [7].

Chromosome number and genome size (GS) are basic cytological characters, normally constant within a species at the same ploidy level [21], although there are exceptions, such as the presence of B-chromosomes [22] or genuine intraspecific GS variation [23, 24]. Chromosome numbers are relatively well documented for *Vaccinium*, as recorded by the Chromosome Counts database [25], where there is data for more than 100 species of the genus. However, GS is only known for a few of its species [12, 26–28]. The nuclear DNA content is a trait correlated with many biological characters such as seed and chloroplast size (positively correlated in most cases [24]), and related to life cycle or environmental factors [29]. In general, plants with smaller GS tend to show faster growth rates and complete faster their life cycle [21, 30]. From an ecophysiological perspective, GS has been correlated to environmental tolerance towards high or low temperatures [31] or soil nutrients [32]. Other studies have even found a relationship between nuclear DNA amount and response to global warming in which plants with smaller genome sizes would be favoured [33]. In general, these associations of GS with plant growth and yield have great potential interest for conservation and breeding purposes on crop species such as *Vaccinium*. This work aims to extend GS information on this genus, which will be ultimately useful for breeding purposes and to analyse its variation considering the phylogenetic context of the studied species.

2. Materials and methods

2.1. Species and sample collection

We obtained samples from 13 *Vaccinium* populations representing five species (*V. corymbosum*, *V. arctostaphylos*, *V. myrtillus*, *V. uliginosum*, and *V. vitis-idaea*) from five different subgenera/sections (*Cyanococcus*, *Hemimyrtillus*, *Myrtillus*, *Vaccinium* and *Vitis-idaea*, respectively). An indication of the collection place as well as data from herbarium vouchers can be found in Table 1. For chromosome counts, young leaf buds were collected during spring and summer and instantly stored in ice and kept overnight. For flow cytometry assays, we sampled fresh young leaves from five individuals per population and stored them at 4°C until they were processed at the laboratory. Leaf tissue employed for DNA sequencing was sampled from the field and immediately stored in silica-gel.

2.2. Chromosome counts

Chromosomes were prepared according to [34] with some modifications. Fixation of leaf buds was performed in methanol/glacial acetic acid (3 : 1) with 1% PVP. The fixative solution was changed several rounds to ensure

Table 1
Provenance and voucher data of the studied populations

Species	Location, collectors, collection date and voucher number
V. arctostaphylos	Kackar Mountains, Rize National Park, Turkey. Sedat Serçe and Nusrat Sultana. 29th June 2018. BC-974075.
V. corymbosum†	Cutivated at the Niğde Ömer Halisdemir University greenhouse (origin of the cultivar line: USA), Turkey. Sedat Serçe and Nusrat Sultana. 21st June 2018. BC-974080.
V. myrtillus (1)	Canillo (1), Vall d'Incles, Andorra. Joan Vallès and Teresa Garnatje. 11th July 2018. BC-974082.
V. myrtillus (2)	Canillo (2), Vall d'Incles, Andorra. Joan Vallès and Teresa Garnatje. 11th July 2018. BC-974081.
V. myrtillus (3)	Devět Skal, Křižánky, Czech Republic. Sònia Garcia, Aleš Kovařík and Daniel Vitales. 29th May 2018. BC-974079.
V. myrtillus (4)	Velke Darko, Škrdlovice, Czech Republic. Sònia Garcia, Aleš Kovařík and Daniel Vitales. 29th May 2018. BC-974078.
V. myrtillus (5)	Rostejn, Trestice, Czech Republic. Sònia Garcia, Aleš Kovařík and Daniel Vitales. 24th May 2018. BC-974077.
V. myrtillus (6)	Mangartska jama, Triglav National Park, Slovenia. Teresa Garnatje and Oriane Hidalgo. 24th July 2018. BC-974071.
V. myrtillus (7)	Kackar Mountains, Rize National Park, Turkey. Sedat Serçe and Nusrat Sultana. 29th June 2018. BC-974074.
V. uliginosum (1)	Canillo, Vall d'Incles, Andorra. Albert Ruzafa 8th July 2018. BCN-E-367.
V. uliginosum (2)	Kackar Mountains, Rize National Park, Turkey. Sedat Serçe and Nusrat Sultana. 29th June 2018. BC-974076.
V. vitis-idaea (1)	Novohradske Hory, Pohorská Ves, Czech Republic. Eva Bártová. 3rd June 2018. BC-974072.
V. vitis-idaea (2)	Velke Darko, Czech Republic. Sònia Garcia, Aleš Kovařík and Daniel Vitales. 29th May 2018. BC-974073.

†cultivar 'Jubilee'.

complete removal of all plant pigments and finally it was preserved at –20°C in the same solution until slide preparation. After removing the outer leaf coat under the microscope, the inner white meristematic portion of a single leaf bud was washed in citrate buffer (4 mM citric acid and 6 mM sodium citrate, pH 4.5) several times to remove the existing fixative and digested overnight at room temperature with the enzyme mixture PINE containing 2% (w/v) cellulase *Aspergillus niger* (Sigma C-1184), 4% (w/v) cellulase Onozuka R10 (Sigma 16419), 2% (w/v) cytohelicase from *Helix pomatia* (Serva C-8274), 0.5% (w/v) pectolyase from *A. japonicus* and 5% (w/v) pectinase from *A. niger* in citrate buffer. The material was digested until it became soft, followed by two cycles of centrifugation and washing in citrate buffer and one centrifugation step in fixative at 4°C at 4500 rpm. The final wash was done also in fixative at 4°C, increased to 5000 rpm. The pellet of nuclei was dissolved in 500 µl of fixative and mixed by slow pipetting. For each slide, 13 µl of mixture were spread, instantly washed in fixative three times and air dried. The air-dried slides were stained with 1% aceto-orcein solution and checked several rounds to ensure perfect staining, which is about 30 minutes. After proper staining, the slide was washed with 70% ethanol to remove extra dye and mounted with Canada balsam solution. A total of 10 well-spread cells, containing metaphase chromosomes, were counted for each species. Slides were analysed using a Leica-Epifluorescence microscope (LEICA, DMIL-LED) equipped with LAS software (Leica) and LEICA high resolution digital camera. Images of well-spread metaphase chromosomes were captured at 100× magnification and analysed. After image acquisition, the final picture was processed through Adobe Photoshop 7 software to balance brightness and contrast, and only using functions that affect the image as a whole.

2.3. Flow cytometry

Five plants per population were studied processing two samples per individual. The internal standards chosen were *Petunia hybrida* 'PxPc6' (2C = 2.85 pg) and *Pisum sativum* 'Express Long' (2C = 8.37 pg) in order to cover the possible ploidy differences with good linearity during the experiment [35]. Nuclei of both internal standard and *Vaccinium* samples were isolated by co-chopping the leaf tissues together in 600 µl of LB01 lysis buffer [36], supplemented with ribonuclease A (RNase A, Boehringer, Meylan, France). The fluorescence measurements were performed in a Gallios flow cytometer (Coulter Corporation; Hialeah, Fla, USA). The instrument was set up with the standard configuration; excitation of the sample was performed by using a blue 488 nm laser. Forward scatter (FSC), side scatter (SSC), and red (620/30 nm) fluorescence (peak and area signals) for propidium iodide (PI) were acquired. Removal of aggregates and background noise was performed by gating red fluorescence area versus red fluorescence peak signals and FSC vs red florescence signal, respectively. Acquisition was stopped automatically at 8,000 nuclei. The lysed cell suspension was filtered through 70 µm nylon mesh to remove cell debris. Samples were incubated in ice until measurement and stained with 36 µl PI solution (1 mg/ml, Sigma-Aldrich, Alcobendas, Madrid) to a final concentration of 60 µg/ml. Flow cytometry measurements were taken twice for each sample and a minimum of 2000 particles per peak were acquired to get a consistent result. Genome sizes (total DNA or 2C-value) of the species were calculated according to the peak position ratios and following the formula: (sample peak mode/internal standard peak mode) * GS (2C) of the standard [37].

2.4. DNA extraction, PCR and sequencing strategy

Either the E.Z.N.A.® Plant DNA Kit (Omega Bio-tek, Inc., Norcross, Georgia, USA) or the CTAB method [38] were used to extract genomic DNA from leaf materials, depending on their quality or available amount. The quality of each extraction was checked with Qubit Fluorometric Quantification (ThermoFisher Scientific, Waltham, Massachusetts, USA). Polymerase chain reaction (PCR) was performed using the T100TM Thermal Cycler (BioRad Laboratories, Hercules, California, USA) in a 25 µL volume following the procedures explained in [24]. Three regions were amplified in this study: one nuclear (Internal Transcribed Spacer of the nuclear ribosomal DNA, ITS) and two chloroplastic (*mat*K and *ndh*F). The ITS region was amplified by PCR using ITS1f and ITS4r primers [39]. The PCR profile used for amplification was 94°C 3 min; 30 × (94°C 20 s; 55°C

1 min; 72°C 1 min); 72°C 10 min [24]. In some cases, the pair of primers 1406f and ITS4r [40] were used when the former did not work. The sequencing primer used for this region was the ITS4r. The *mat*K region was amplified by PCR using 1848f and 710r primers [41]. The *ndh*F region was amplified by PCR using two primer pairs; for the first segment we used the 90F (5′ CGT ATC TGG GCT TTT CTA AGT G 3′) and 912R (5′ GAG CAA GTG CTA AAG TAG CTC CTA A 3′) (both specifically designed), and for the second segment we used the primers 803F and 1318R [42]. The PCR profile used for *mat*K and *ndh*F amplification was described in [43]. Sequencing was performed with the Big Dye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, California, USA) at the Serveis Científics i Tecnològics (Universitat de Barcelona) on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, California, USA). Due to the length of the fragments amplified in both regions, we used the reverse and forward primers of each segment for the sequencing reaction. GenBank accession numbers for these sequences are MN134402-MN134402 (ITS), MN150130-MN150135 (n*dh*F) and MN150136-MN150141 (*mat*K).

2.5. Phylogenetic and statistical analyses (including reconstruction of ancestral GS)

The chloroplast (*mat*K and *ndh*F) and nuclear (ITS) datasets were analysed separately since loci from different genomic compartments may undergo independent evolution, potentially generating incorrect phylogenetic inferences based on concatenated datasets [44]. The cpDNA dataset contained 68 taxa with a sequence length of 2820 characters (1–1543 *mat*K and 1544–2820 *ndh*F). The nrDNA dataset contained 68 taxa with a sequence length of 694 characters. The two matrices were aligned using MAFFT [45] and finally adjusted manually on Geneious Prime v2019.1.3. Non-sequenced fragments were scored as missing data (N). In total, the matrices included DNA data from 65 (nrDNA) and 64 (cpDNA) taxa, where five of them have been sequenced for the first time in this study and the remaining were obtained from Genbank (Table S1). The two datasets were analysed using Bayesian Inference (BI), previously fitting the best evolutionary model with MEGA X [46], independently for the nuclear and chloroplast matrices. For both datasets, the best evolutionary model fitted was the GTR + G + I. For the BI analyses, the program MrBayes v3.2.1 [47] was used to run two independent Markov chains Monte Carlo (MCMC) for 50,000,000 generations for each dataset, with tree sampling every 1,000 generations. The average standard deviation of the split frequencies was checked to be less than 0.01 and the potential scale reduction factor was near 1.0 for all parameters. The first 25% of the trees were discarded as 'burn-in' and the posterior probabilities were estimated constructing the 50% majority rule consensus tree, using as outgroup the Vaccinieae tribe species *Gaultheria procumbens, Leucothoë fontanesiana, Zenobia pulverulenta* and *Andromeda polifolia* [3, 16].

To analyse the evolution of GS in *Vaccinium*, we performed the ancestral character reconstruction of GS values using unordered maximum parsimony implemented for continuous characters in Mesquite v.3.6 software [48]. First, we selected the species with available genome size and sequencing data for the molecular markers (cpDNA and nrDNA) mentioned above, either generated on this work or obtained from online repositories (i.e. Genbank) as well as published papers (Tables 2 and 3). The trees used as the input files for ancestral character reconstructions were generated by BI using only the species with available GS and sequence data (15 taxa, with *Gaultheria procumbens* set as outgroup), fitting the best evolutionary model with MEGA X. Finally, using these reduced phylogenetic reconstructions based on nrDNA and cpDNA, ancestral state inference was calculated for holoploid (2C) GS.

3. Results

Chromosome counts were performed for all the studied species in order to verify the ploidy level of the accessions, except for *V. vitis-idaea* because of unavailability of the appropriate tissue. Two different ploidy levels (diploid and tetraploid) were detected. *Vaccinium myrtillus* and *V. uliginosum* were diploid with chromosome

Table 2
Chromosome number and genome size of studied *Vaccinium* species. Standard deviation was calculated from the measurement of five different individuals, in most cases, each measured twice

Species	Section	Location	2n	PL	GS (2C pg) ± SD	1Cx	Internal standard
V. arctostaphylos	*Hemimyrtillus*	Turkey	48*	4x	3.13 ± 0.07	0.78	*Pisum sativum*
V. corymbosum†	*Cyanococcus*	Turkey	48*	4x	3.17 ± 0.18	0.79	*Pisum sativum*
V. myrtillus (1)	*Myrtillus*	Andorra	24	2x	1.28 ± 0.13	0.64	*Petunia hybrida*
V. myrtillus (2)	*Myrtillus*	Andorra	24	2x	1.16 ± 0.11	0.58	*Petunia hybrida*
V. myrtillus (3)	*Myrtillus*	Czech Republic	24	2x	1.26 ± 0.04	0.63	*Petunia hybrida*
V. myrtillus (4)	*Myrtillus*	Czech Republic	24	2x	1.24 ± 0.06	0.62	*Petunia hybrida*
V. myrtillus (5)	*Myrtillus*	Czech Republic	24	2x	1.20 ± 0.03	0.60	*Petunia hybrida*
V. myrtillus (6)	*Myrtillus*	Slovenia	24	2x	1.30 ± 0.03	0.65	*Petunia hybrida*
V. myrtillus (7)	*Myrtillus*	Turkey	24*	2x	1.27 ± 0.06	0.64	*Petunia hybrida*
V. uliginosum (1)	*Vaccinium*	Andorra	24	2x	1.47 ± 0.11	0.74	*Petunia hybrida*
V. uliginosum (2)	*Vaccinium*	Turkey	24*	2x	1.34 ± 0.02	0.67	*Petunia hybrida*
V. vitis-idaea (1)	*Vitis-idaea*	Czech Republic	24	2x	1.28 ± 0.03	0.64	*Petunia hybrida*
V. vitis-idaea (2)	*Vitis-idaea*	Czech Republic	24	2x	1.24 ± 0.03	0.62	*Petunia hybrida*

†cultivar 'Jubilee'. *chromosome counts obtained in the present study; the other counts obtained through the Chromosome Counts Database [25]

number 2n = 2x = 24 and *V. corymbosum* ('Jubilee' cultivar) and *V. arctostaphylos* were tetraploid with chromosome number 2n = 4x = 48 (Table 2 and Fig. 1). Genome size estimations were performed for all the studied materials (Table 1). The average half peak coefficient of variation (HPCV%) was 1.11% and 3.44% for internal standards and studied samples, respectively. For both *V. corymbosum* and *V. arctostaphylos* we only estimated the GS of one population, while seven populations were analysed for *V. myrtillus* (GS ranging from 1.16 pg to 1.30 pg; i.e. 10.77% of intraspecific variation), two for *V. uliginosum* (GS ranging from 1.34 pg to 1.47 pg; i.e. 9.70% intraspecific variation) and two for *V. vitis-idaea* (GS ranging from 1.24 pg to 1.28 pg; i.e. 3.22% intraspecific variation). Average monoploid GS (1Cx) was 0.63 pg for diploids and 0.79 pg for tetraploids. The relationship between GS and ploidy level was analysed considering previously published data in the genus (Table 2). A positive significant relationship between holoploid GS (2C) and ploidy level was found (Pearson's $r = 0.94$, DF = 21, $p < 0.0001$), while the relationship between monoploid GS (1Cx) and ploidy level was non-significant (Pearson's $r = -0.14$, DF = 21, $p = 0.4983$).

In order to place the studied species in a molecular systematic background we have reconstructed the phylogenetic history of the genus including these taxa (i.e. published phylogenetic reconstructions of *Vaccinium* did not include all the species here studied). Both reconstructions showed congruent results in the position and structure of many phylogenetic groups but there were also certain inconsistences regarding the placement of some species and clades. Both the nrDNA and the cpDNA trees supported the monophyly of tribe Vaccinieae but not that of genus *Vaccinium*, since species from several other genera (i.e. *Cavendishia, Paphia, Notoptora*, etc.) appear mixed in the clades containing most *Vaccinium* species. According to the reconstruction based on cpDNA (Figure S1), all the newly sequenced accessions were placed together with previously sequenced specimens of the same species. However, in the tree based on nrDNA (Figure S2), the new accessions of *V. corymbosum* 'Jubilee' and *V. uliginosus* we analysed in this study were split in different clades from previously sequenced specimens of the same species. In this nrDNA phylogenetic inference, the specimens of *V. uliginosus* from Andorra and Turkey were placed in a clade (PP = 0.97) with *V. vitis-idaea* and *V. macrocarpon*, while a previously sequenced accession of *V. uliginosum* (from Canada [49]) appeared in a clade (PP = 0.99) with *V. arctostaphylos*, *V. cylindraceum* and *V. padifolium*. Based on the same nrDNA reconstruction, the accession of *V. corymbosum* 'Jubilee' we sequenced

Table 3
Genome size value published so far for different taxa of the family Ericaceae in different individual studies. pg = Picogram, Mbp = Mega base pairs, FC = Flow cytometry, PI = Propidium Iodide, DAPI = 4′,6-diamidino-2-phenylindole, NGS = data coming from a next generation sequencing project. Superscript numbers after species names indicate the source publication in the literature list

Taxa	2n	Ploidy level	Estimation method	2C (pg)	1Cx (pg)
Genus *Vaccinium*					
Vaccinium boreale[26]	24	2	FC:PI	1.18	0.59
V. corymbosum[26]	24	2	FC:PI	1.33	0.67
V. darrowi[26]	24	2	FC:PI	1.31	0.66
V. elliottii[26]	24	2	FC:PI	1.26	0.63
V. myrtilloides[26]	24	2	FC:PI	1.26	0.63
V. pallidum[26]	24	2	FC:PI	1.21	0.61
V. tenellum[26]	24	2	FC:PI	1.3	0.65
V. macrocarpon[28]	24	2	NGS	1.16	0.58
V. arboreum[12]	24	2	FC:PI	1.02	0.51
V. crassifolium[12]	24	2	FC:PI	1.1	0.55
V. darrowii[12]	24	2	FC:PI	1.09	0.55
V. elliottii[12]	24	2	FC:PI	1.05	0.53
V. pallidum[12]	24	2	FC:PI	1.1	0.55
V. stamineum[12]	24	2	FC:PI	1.01	0.51
V. angustifolium[12]	48	4	FC:PI	2.02	0.51
V. arboreum[12]	48	4	FC:PI	1.98	0.50
V. corymbosum[12]	48	4	FC:PI	2.11	0.53
V. myrsinites[12]	48	4	FC:PI	2.11	0.53
V. pallidum[12]	48	4	FC:PI	2.1	0.53
V. pensylvanicum[12]	48	4	FC:PI	1.95	0.49
V. virgatum[12]	72	6	FC:PI	3.8	0.63
V. corymbosum 'Jubilee'[12]	48	4	FC:PI	2.1	0.53
V. myrtilloides[27]			FC:PI	1.29	
V. angustifolium[27]			FC:PI	2.07	
Genus *Arctostaphylos*					
A. uva-ursi[57]			FC:PI	2.49	
Genus *Empetrum*					
E. hermaphroditum[58]	52	4	FC:PI	2.56	0.64
E. nigrum[58]	26	2	FC:PI	1.29	0.65
Genus *Erica*					
E. multiflora[59]			FC:PI	0.95	
Genus *Chimaphila*					
Chimaphila umbellata[27]			FC:PI	18.2	
Genus *Gaultheria*					
Gaultheria procumbens[27]			FC:PI	2.5 to 3.8	
G. baccata[27]			FC:PI	1.1 to 1.3	

(*Continued*)

Table 3
(*Continued*)

Taxa	2n	Ploidy level	Estimation method	2C (pg)	1Cx (pg)
Genus *Monotropa*					
Monotropa uniflora[27]			FC:PI	59.8	
Genus *Pyrola*					
Pyrola elliptica[27]			FC:PI	9.6 to 9.3	
Genus *Rhododendron*					
Rhododendron delavayi[60]			NGS	1.42	
R. catawbiense[54]		2	FC:DAPI	1.44	0.72
R. fortunei[54]		2	FC:DAPI	1.55	0.78
R. maximum[54]		2	FC:DAPI	1.44 to 1.53	0.72
R. ponticum[54]		2	FC:DAPI	1.46	0.73
R. sinogrande[54]		2	FC:DAPI	1.54	0.77
R. edgeworthii[54]		2	FC:DAPI	1.75	0.88
R. augustinii[54]		4	FC:DAPI	3.1	0.78
R. maddenii[54]		6	FC:DAPI	4.39 to 4.47	0.73
R. maddenii[54]		8	FC:DAPI	5.42 to 5.97	0.68
R. alabamense[54]		2	FC:DAPI	1.66	0.83
R. arborescens[54]		2	FC:DAPI	1.65	0.83
R. austrinum[54]		2	FC:DAPI	1.59 to 1.64	0.80
R. canescens[54]		2	FC:DAPI	1.61 to 1.72	0.81
R. cumberlandense[54]		2	FC:DAPI	1.63	0.82
R. eastmanii[54]		2	FC:DAPI	1.58 to 1.60	0.79
R. flammeum[54]		2	FC:DAPI	1.68 to 1.72	0.84
R. occidentale[54]		2	FC:DAPI	1.51	0.76
R. triploides[54]		2	FC:DAPI	2.3 to 2.52	1.15
R. atlanticum[54]		4	FC:DAPI	3.01 to 3.33	0.75
R. austrinum[54]		4	FC:DAPI	3.11 to 3.88	0.78
R. stenopetalum[54]		2	FC:DAPI	1.27	0.64

was placed in an early-diverging clade with *V. darrowii*, *V. angustifolium*, *V. hirsutum* and *V. myrsinites*, whereas the formerly sequenced specimen of this species (coming from a wild population) occupied a poorly supported but phylogenetically distant position from that clade. Regarding the phylogenetic relationships among *Vaccinium* groups of species, the tree generated from the analysis of ITS sequences (Figure S1) was considerably more resolved than the one coming from chloroplast DNA (Figure S2).

Ancestral character reconstructions of GS values was limited to 16 taxa of the genus, some species being only present in the reconstruction based on nrDNA (i.e. *V. darrowii*; *V. boreale*) or in the one based on cpDNA (i.e. *V. mirtylloides*), depending on sequence data availability. The reduced evolutionary reconstruction based on rDNA sequences showed good resolution for most of the nodes (PP > 0.95; Fig. 2) and the systematic structure was congruent with the ITS tree of Vaccinieae taxa (Fig. S1) as well as with previous phylogenetic treatments of the tribe [3, 16]. In contrast, as occurred in the cpDNA tree of Vaccinieae (Fig. S2), the ancestral GS phylogenetic reconstruction based on cpDNA data (Fig. 2) showed poor resolution in most of the nodes of the tree. The ancestral state inference indicates a GS reduction for *Vaccinium* with respect to the outgroup (*Gaultheria*). According to

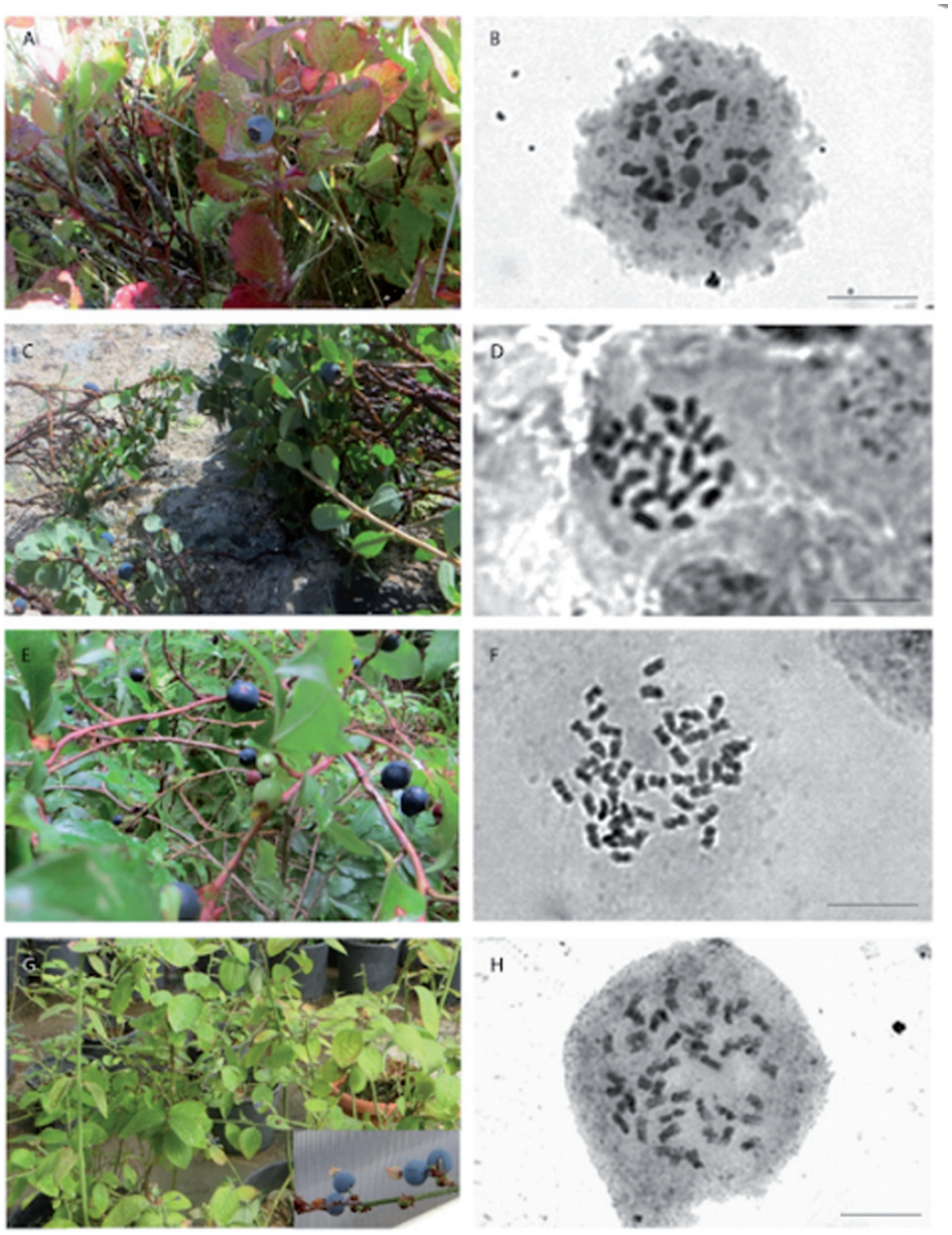

Fig. 1. Pictures and chromosome number of the studied *Vaccinium* species. (A-B) *V. myrtillus* (2n = 2x = 24); (C-D) *V. uliginosum* (2n = 2x = 24); (E-F) *V. arctostaphylos* (2n = 4x = 48); (G-H) *V. corymbosum* (cultivar 'Jubilee') (2n = 4x = 48), with detail of the fruit in the lower right corner. Scale bars 10 μm.

both nrDNA and cpDNA reconstructions, several independent shifts of GS within the genus have occurred, most of them related to changes in ploidy level of the species. The reconstructed ancestral GS inference for the MRCA of the genus ranged between 1.88 to 2.31 pg for 2C values.

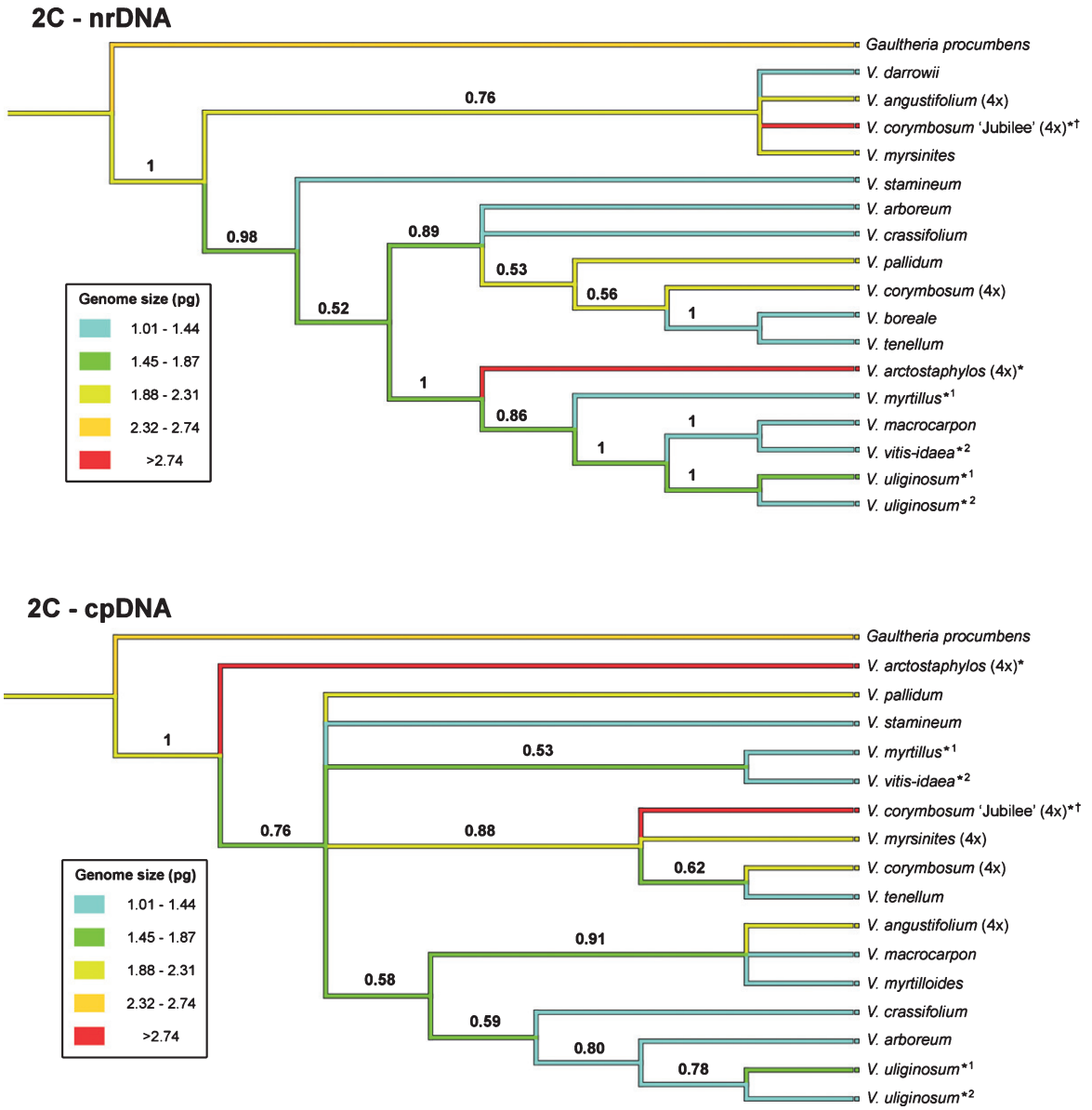

Fig. 2. Reconstruction of the ancestral GS (2C) values in the studied species of *Vaccinium* based on nrDNA and cpDNA phylogenetic inferences. *Gaultheria procumbens* was used as an outgroup. Values above branches indicate posterior probabilities (PP). Sequences obtained for this study indicated by an asterisk. Numbers in superscripts indicate the provenance of populations (as in Table 1).

4. Discussion

The study has confirmed previously known chromosome numbers for all the studied species excepting for *V. vitis-idaea*, for which material for chromosomal analysis was not available. According to the Chromosome Counts Database [25] the obtained counts agree with the most commonly found numbers for *V. corymbosum* (in which the tetraploid count 2n = 48 is much more common than the exceptionally found 2n = 24, 36, 54, 56,

60, 88 and 72), *V. arctostaphylos* (in which only the tetraploid count 2n = 48 has been found up to date) and *V. myrtillus* (in which, although 2n = 24 is the overall most common, there are exceptional counts of 2n = 20 and 48). In the case of *V. uliginosum*, we counted 2n = 24 in the accession from Turkey, while both 2n = 24 and 48 are similarly found in previous studies (although rare counts of 2n = 39, 45 and 72 have also been reported). The accessions of *V. uliginosum* from the Czech Republic and from Andorra (the last one also being diploid according to our GS estimates) are placed together in our nrDNA phylogenetic reconstruction (Fig. S1) (which is overall consistent with those of [3, 16] on the tribe and genus) but in a different clade from the previously sequenced samples obtained from Genbank. Phylogenetic split among diploid and tetraploid individuals of *V. uliginosum* was already reported by [50], interpreting this pattern as a result of hybridization, polyploidization and homogenization processes during the complex evolutionary history of the species. As mentioned previously, we could not assess chromosome number for any of our populations of *V. vitis-idaea*, but the GS values we obtained indicate that both analysed populations are most likely diploid. Indeed, for this species, all extant counts are 2n = 24 except a single one reporting a triploid accession (2n = 36) [25]. However, whenever possible, it is recommended to perform chromosome counts on the same populations in which GS will be assessed to ascertain ploidy, rather than inferring it from previously known data, since intraspecific variation can exist at the chromosome level, involving different ploidy levels, the presence of B-chromosomes, aneuploidy or disploidy.

This work contributes to the first GS data for three species which are relatively common in mostly cold (*V. vitis-idaea* and *V. uliginosum*) or temperate (*V. arctostaphylos*) regions in the Northern hemisphere, the former two including populations in Eurasia and North America. Both at diploid and tetraploid levels our new data extend the range of GS from the upper side, contributing the highest GS for diploid *Vaccinium* to *V. uliginosum* (2C = 1.47 pg) and the highest GS for tetraploid *Vaccinium* to *V. corymbosum* 'Jubilee' (2C = 3.16 pg). Even if we do not consider this cultivar, in which hybridization or other domestication processes could have been involved (see comment on this below), the second highest GS in the genus for a tetraploid species also belongs to our newly assessed *V. arctostaphylos* (2C = 3.13 pg). Our study also provides data for new populations of the economically important and widely distributed species *V. myrtillus.* In this case, we measured geographically distant populations from Andorra, Turkey and the Czech Republic, while the only GS assessment to date (2C = 1.17 pg) [51] belonged to an accession from Bosnia and Herzegovina. Our data, ranging from 2C = 1.16–1.28 pg are consistent with previous measurements of *V. myrtillus*, indicating a narrow range of GS variation irrespective of the geographical distribution of the populations analysed. Low intraspecific variation has also been found for both *V. uliginosum* and *V. vitis-idaea*. Only in the case of *V. corymbosum* 'Jubilee' have we found a remarkable degree of variation with respect to previously known GS estimates, our measures being 50.48% larger than the previously assessed [12]. The different GS values could be explained by the fact that this is one of the most popular and widely extended blueberry cultivars – because of its ornamental and food value – in which hybridization processes for breeding purposes are likely involved [8]. Indeed, the incongruent phylogenetic position of *V. corymbosum* 'Jubilee' between cpDNA and nrDNA reconstructions (Figs. S1 and S2) supports the hybridization hypothesis.

The known GS range for *Vaccinium* species (from 2C = 1.01 to 1.47 pg at the diploid level and from 2C = 1.95 to 3.17 pg at the tetraploid level) is quite narrow and using Leitch et al. [52] categories (so-called Leitch's criteria) all species would belong to the group of "very small genomes" (2C $\leq$ 2.8 pg) except our tetraploids *V. corymbosum* and *V. arctostaphylos* and the hexaploid *V. virgatum* (2C = 3.8 pg, [12]) which would be considered just "small genomes" (2C = 2.9–7 pg). It should be noted that, although the known range may not reliably reflect the real GS variation within the genus, the species with known GS belong to nine sections out of the 30 within the genus [1], so although scarce, the extant data could barely represent the probably small GS dominant in the genus and its low diversity within *Vaccinium*. The reconstruction of the ancestral GS state in the genus (2C = 1.88–2.31 pg), which needs to be taken with caution given the scarcity of GS data for *Vaccinium* species (genome size estimated for 19 taxa for a genus with *ca.* 500 species, and only 16 could be used for the reconstruction), also points to "very small" GS according to Leitch's categories. A very small genome size is considered the ancestral condition, as

well as the most common, in flowering plants [52]. Within the genus (considering diploid GS) there have been scattered events leading to an increased GS in certain species (see Fig. 2). Although their GS would still fall within the "very small" category, these mild changes reflect the dynamic nature of GS evolution, even in groups with a narrow range of variation.

A common response to polyploidy is genome downsizing [53] in which whole genome duplication (WGD) is not accompanied by a proportional GS increase. The available data for *Vaccinium* GS and ploidy levels indicates a slight genome downsizing from the diploid to the tetraploid level (average 1Cx = 0.60 pg for diploids and average 1Cx = 0.56 pg for tetraploids), however, the only hexaploid with available data (1Cx = 0.63 pg) points to a genome upsizing for this ploidy level. Conversely, the available data for the Ericaceae genus *Rhododendron* [54] points to a clear and global genome downsizing in which 1Cx is gradually reduced across ploidy levels. However, it would be necessary to have more GS data at the genus level in order to properly assess the extent of genome ups and downs in *Vaccinium*, since such differences would be better assessed between closely related species, and our dataset is quite scanty.

Genome size data availability for family Ericaceae are even more scattered than for the genus *Vaccinium*. Despite being one of the largest plant families with more than 4000 species and around 125 genera [16], up to our knowledge we only have GS for a few species from genera *Arctostaphylos* (1/*ca*. 60), *Empetrum* (2/3-18), *Erica* (1/*ca*. 900), *Chimaphila* (1/5), *Gaultheria* (2/*ca*. 135), *Monotropa* (1/2), *Pyrola* (1/*ca*. 30) and *Rhododendron* (21/*ca*. 1000). Table 3 shows the known GS in family Ericaceae together with the source publications, which would represent roughly 1% of Ericaceae with known GS. Apparently, genus *Vaccinium* is one of the best represented (among the genera with a relatively high number of species), and genus *Rhododendron* is similarly known. Both genera are more common in temperate or even cold regions of the Northern hemisphere (although both have tropical species). Certainly, the large diversity of both genera, their widespread distribution and their economic interest (either as edible/medicinal for *Vaccinium* or mostly ornamental for *Rhododendron*) may have played a role on relative GS data abundance of these taxa. Despite being not too closely related from the phylogenetic point of view [55], both share similar GS ranges, similar base chromosome number ($x = 12$ in *Vaccinium* and $x = 13$ in *Rhododendron*) and ploidy levels. However, other Ericacaceae genera (i.e. *Monotropa*, *Pyrola* and *Chimaphila*) show contrastingly higher GS values (see Table 3). In the case of *Monotropa uniflora*, the parasitic nature of this species could be involved in its extremely large genome, as found previously in other parasitic plants (e.g. *Viscum album* [56]).

5. Conclusions

This study provides new GS data for three *Vaccinium* species and contributes additional GS information for two more, all of them can be considered as "very small", and an apparent GS reduction has occurred in the genus; altogether GS are known for about 4% of the genus when considering previous data. We also confirm the most common ploidy levels for the assessed species, namely 2x and 4x. Most of the assessed *Vaccinium* species have economic interest as food and ornamentals or are wild relatives of important crops in this genus such as *V. myrtillus*, for which this study extends its known GS range of variation, or *V. corymbosum*, one of the most widely cultivated blueberry species both for edible and ornamental uses. We have also discussed GS variation and ancestral state reconstruction considering the phylogenetic framework of the studied species, although the scarcity of GS data available for the genus limits the extent of its conclusions, and a more detailed study covering a larger taxonomic diversity of the genus would be desirable. Finally, the amount of genomic research data for *Vaccinium* is steadily increasing [Genome Database for *Vaccinium*: https://www.vaccinium.org/]. Given that GS is an essential prior information to consider in any study concerning whole genome sequencing, this information will be useful in any genomic approach that may be attempted for these taxa.

Conflict of interest

The authors have no conflict of interest to report.

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