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Genome size variation and polyploidy incidence in the alpine flora from Spain

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Abstract

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The interest to study genome evolution, in particular genome size variation and polyploid incidence, has increased in recent years. Still, only a few studies have been focused at a community level. Of particular interest are high mountain species, because of the high frequency of narrow endemics and their higher susceptibility to extinction due to the effects of climate change. In the present study we explored genome size variation and polyploidy incidence in the entomophilous plant communities of two distinct mountain ranges, the Sierra Nevada and Picos de Europa National Parks. For that, chromosome numbers and DNA ploidies were assessed through a review of the literature, and the genome size and incidence of polyploidy in 39 taxa from several key genera were estimated using flow cytometry. In this study, first genome size estimations are given for 32 taxa. The majority of the analyzed taxa presented very small to small genome sizes (2C ≤ 7.0 pg), with no differences being detected between genome size and geographic origin and distribution ranges. A low incidence of polyploid taxa was observed (23.3%), with polyploids being more common in Picos de Europa than in Sierra Nevada. Most taxa inferred as polyploids were high altitude plants, but no clear pattern between polyploidy incidence and endemic status was observed. The obtained results are discussed within the context of angiosperm's genome size variation and of polyploidy incidence in alpine and arctic flora, contributing to the scientific knowledge of these natural communities of great biological importance.

Keywords: alpine vegetation, DNA ploidy level, nuclear DNA content, Picos de Europa, Sierra Nevada.

Resumen

Loureiro, J., Castro, M., Cerca de Oliveira, J., Mota, L. & Torices, R. 2013. Variación en el tamaño del genoma e incidencia de la poliploidía en la flora alpina española. *Anales Jard. Bot. Madrid* 70(1): 39-47 (en inglés).

El interés en el estudio de la evolución del genoma, especialmente de la variación en tamaño y de la incidencia de poliploidía, se ha incrementado en los últimos años. Sin embargo, sólo unos pocos estudios se han centrado en el nivel de comunidades. Las especies de alta montaña son especialmente interesantes debido a la alta frecuencia de especies endémicas y a que son consideradas muy susceptibles a la extinción por los efectos del cambio climático. En el presente estudio, exploramos la variación en el tamaño genómico y la incidencia de poliploidía en las comunidades de plantas entomófilas de alta montaña de dos macizos montañosos: el Parque Nacional de Sierra Nevada y el Parque Nacional de Picos de Europa. Para ello, se evaluó el número de cromosomas y el nivel de ploidía por medio de una revisión bibliográfica, mientras que el tamaño genómico y la incidencia de poliploidía se estimaron en 39 taxones de varios géneros usando citometría de flujo. En este estudio, se proporcionan las primeras estimaciones del tamaño genómico para 32 taxones. La mayoría de los taxones analizados presentaron tamaños genómicos pequeños o muy pequeños (2C ≤ 7.0 pg), sin mostrar diferencias en el tamaño genómico asociadas a su origen geográfico o rango de distribución. Se observó una baja incidencia de taxones poliploides (23.3%), siendo éstos más comunes entre las plantas de Picos de Europa que entre las de Sierra Nevada. La mayor parte de los taxones considerados como poliploides fueron plantas restringidas a las montañas, sin embargo no se observó un patrón claro entre la incidencia de poliploidía y el grado de endemismo. Los resultados obtenidos son discutidos dentro del contexto de variación en el tamaño del genoma y de la incidencia de poliploidía en las floras árticas y alpinas, contribuyendo al conocimiento científico de estas comunidades naturales de gran importancia biológica.

Palabras clave: contenido nuclear de ADN, nivel de ploidía, Picos de Europa, Sierra Nevada, vegetación alpina.

INTRODUCTION

The study of the genome size and its variation has been increasingly important in many areas of plant research, including taxonomy, biosystematics, ecology and population biology. Nuclear DNA amount (C-value) has been referred as an important biodiversity character, whose study provides a strong unifying element in biology with practical and predictive uses (Bennett & Leitch, 2005). Despite of the increase in the number of genome size estimates over the years, no records are still available for approximately 97.5% of the angiosperms (Bennett & Leitch, 2012). Still, the available data for approximately 7500 species, already evidenced a large variation in genome size, spanning nearly a 2500-fold range, with *Genlisea margaretae* Hutch. (Lentibulariaceae, 1C = 0.06 pg; Greilhuber & al., 2006) and *Paris japonica* Franch. (Melanthiaceae, 1C = 152.20 pg; Pellicer & al., 2010) representing the smallest and the largest genomes discovered so far.

Genome evolution is now considered to be a highly dynamic process and its size results from a balance between expansion and contraction forces (increasing and decreasing its size, respectively; Bennett & Leitch, 2005). In homoploid plants (i.e., species with the same number of chromosomes), genome expansion has been attributed to amplification and insertion of transposable genetic elements (Ma & al., 2005; Vitte & Bennetzen, 2006) and/or gain of chromosome regions, such as tandem repeats (Lim & al., 2006). By other way, genome contraction has been associated with deletional mechanisms, such as, unequal intra-strand homologous recombination, illegitimate recombination and/or higher rate of nucleotide deletion over insertion (Swigonova & al., 2005).

Another important mechanism responsible for rapid increases in genome size is polyploidy. Indeed, the most recent

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estimations suggest that up to 100% of angiosperms have experienced one or more episodes of polyploidization during their evolutionary history (Wood & al., 2009). Polyploids arise most frequently by the fusion of unreduced gametes, and may result either from the doubling of a single genome (autopolyploidy) or by the combination of two or more distinct, yet related, genomes (allopolyploidy) (Grant, 1981). Due to the possibility of immediate changes in the phenotype, fitness and ecological tolerances of polyploid lineages in comparison with its diploid progenitors, polyploidization has been proposed as a major mechanism of sympatric speciation (Adams & Wendel, 2005), and might allow evolutionary transitions that would have been previously impossible (Comai, 2005; Hegarty & Hiscock, 2008). Thus, in a single genetic event, polyploidy may produce a broad variation, increasing the likelihood of coping with drastic environmental stress, such as climate changes (Fawcett & Van de Peer, 2010).

It is generally considered that polyploids are more frequent at higher latitude or altitude than related diploids (Petit & Thompson, 1999). This pattern is based on two hypotheses: first, polyploids might be more successful than diploids in colonizing after glaciation (Brochmann & al., 2004); second the last glacial maximum climate enforced range shifts that might have led to the formation of hybrids of formerly allopatric taxa, which subsequently suffered chromosome doubling to restore fertility [secondary contact hypothesis of Stebbins (1984)]. Indeed, alpine and arctic floras have been reported to have high frequencies of polyploids (Abbott & Brochmann, 2003; Brochmann & al., 2004; Nie & al., 2005).

In Europe, it has been forecasted that approximately 60% of mountains species could go extinct because of their disproportional sensitivity to climate change (Thuiller & al., 2005). Furthermore, the greatest impacts are expected to occur in the transition between the Mediterranean and Euro-Siberian regions (Thuiller & al., 2005), areas of high conservation interest. As part of an ongoing project focused on the study of pollination interactions in high mountain plants (Santamaría & al., 2011a, b), in the present study we explored genome size variation and polyploidy incidence on two distinct mountain ranges in Spain, placed in the Mediterranean region and in its transition with the Euro-Siberian region, the Sierra Nevada and Picos de Europa National Parks, respectively. For that we: (i) compiled chromosome numbers and DNA ploidy level through an exhaustive review of the bibliographic literature; and (ii) estimated the genome size and incidence of polyploidy in a diverse array of taxa from several key genera of the entomophilous plant community. Considering that polyploid individuals may have higher colonization success after glacial periods than their diploid progenitors (Brochmann & al., 2004), we expect that the high mountain communities studied will show a high frequency of polyploids.

MATERIAL AND METHODS

Plant material and study sites

We explored some genome traits in two high mountain plant communities from two distinct mountain ranges. Both communities were characterized by growing above the tree line (altitudinal tree limit). In the Picos de Europa National Park, we surveyed the plant community in the "Jou de los Cabrones" at 2050 m a.s.l. (43° 12' 50.60" N, 4° 51' 27.08 W); whereas in the Sierra Nevada National Park plants were sampled in the "Borreguil de San Juan" at 2900 m a.s.l.(37° 04' 19.88 N; 3° 22' 26.13 W). We collected plant samples from 39 taxa (as for species or infraspecific categories): 23 from Picos de Europa, and 16 from Sierra Nevada (Table 1). Field collections were carried out during the flowering season (July to August) of the studied taxa. In each site, leaves from up to 50 individuals were collected randomly and stored in plastic bags. During sample transportation into the laboratory, samples were kept in cold conditions and after arrival they were maintained at 4 ºC until use (up to three days). All plant material was identified to species or subspecies level with the exception of one taxon belonging to the genus *Hieracium.* Given the problems associated with *Hieracium* taxonomy and, in particular, with the endemic species from Cantabrian range (Mateo Sanz, 1996) we used the broader group of *Hieracium* gr. *mixtum* for referring to the collected material of this genus. Voucher specimens were collected and are available through the Herbarium of the University of Coimbra (COI).

Data on species distribution, in particular if the studied taxa were distributed only in mountain areas (*mountain specialist*) or widely distributed, and if they were endemic either to Sierra Nevada or Picos de Europa mountain ranges or not were acquired using *Flora iberica* (Castroviejo & al., 1986-2012), *Flora Vascular de Andalucía Oriental* (Blanca & al., 2001), Anthos (Aedo & Castroviejo, 2005) and the Global Biodiversity Information Facility (http://data.gbif.org; http://data.gbif.org). For Picos de Europa community, we also considered as narrow endemics those plants that were distributed in both Picos de Europa and Pyrenees, such as *Minuartia villarsii*, and *Saxifraga hirsuta* subsp. *paucicrenata*.

Genome size estimations

Genome size was estimated using flow cytometry following the method of Galbraith & al. (1983). In brief, approximately 50 mg of leaves of the sample species and of the internal reference standard were chopped with a razor blade in a glass Petri dish containing 1 mL of WPB buffer (0.2 M Tris.HCl, 4 mM $MgCl_2.6H_2O$, 1% Triton X-100, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% PVP-10, pH adjusted to 7.5 and stored at 4 ºC; Loureiro & al., 2007). Nuclear suspensions were then filtered through an 50 µm nylon filter and 50 $\mu g.mL^{-1}$ of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 $\mu g.mL^{-1}$ of RNAse (Fluka, Buchs, Switzerland) were added to sample tubes to stain the DNA and avoid staining of double stranded RNA, respectively. Samples were kept at room temperature and were analyzed within a 5 min period in a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a 532 nm green solid-state laser, operating at 30 mW. Integral fluorescence and fluorescence height and width emitted from nuclei were collected through a 620 nm band-pass interference filter. After the analysis of the first sample of each taxon, the amplifier system was set to a constant voltage and gain. Each

day, prior to analysis, the overall instrument quality was assessed using PI stained nuclei of *Pisum sativum* L. 'Ctirad'.

Relative fluorescence intensity (FL) histograms were obtained and evaluated using FloMax software v2.5 (Partec GmbH, Münster, Germany). Furthermore, FL vs. time and FL vs. side light scatter in logarithmic scale cytograms were also obtained. In the latter cytogram, a region of interest comprising mostly the isolated nuclei was defined. The FL histogram in linear scale was gated with this region. At least 1,500 nuclei in both sample's and standard's G_1 peaks were analyzed per sample (Suda & al., 2007).

For each taxon, ploidy level and genome size was obtained for 5 (rarely 3) individuals. For the remaining individuals, only ploidy level information was gathered as the pooled samples strategy was followed. This strategy consisted of analyzing the leaf tissue of up to 5 individuals plus the reference standard at the same time. If multiple peaks arose, samples were repeated to determine exactly how many individuals with different ploidy level were present in the pooled sample.

The holoploid genome size in pg (2C; *sensu* Greilhuber & al., 2005) of each individual was estimated using the following formula:

$$GS_s = G_{1s} / G_{1r} \times GS_r$$

where GS_s and GS_r are the genome size of sample and reference nuclei respectively, and G_{1s} and G_{1r} are the mean G_1 fluorescence of sample and reference nuclei respectively.

The following reference standards were grown from seeds: *Raphanus sativus* L. 'Saxa' (2C = 1.11 pg; Doležel & al., 1998); *Solanum lycopersicon* L. 'Stupické' (2C = 1.96 pg; Doležel & al., 1992); *Pisum sativum* 'Ctirad' (2C = 9.09 pg; Doležel & al., 1998); *Vicia faba* L. 'Inovec' (2C = 26.90 pg; Doležel & al., 1992). Seeds were sown in plastic pots filed with commercial peat and pots were kept in a climate chamber operating at 20 ± 2 ºC, with a photoperiod of 16h/8h (light/dark) and a light intensity of 530 ± 2 µE/m²/s.

DNA ploidy level estimations

In order to estimate the DNA ploidy level (*sensu* Suda & al., 2006) an extensive bibliographic review on the known ploidy levels, chromosome counts, basic chromosome number and genome size of the studied taxa was performed. For chromosome counts the following bibliography and online databases were mostly used: Index of Plant Chromosome Numbers (Goldblatt & Johnson, 1976-), *Flora iberica* (Castroviejo & al., 1986-2012), Anthos (Aedo & Castroviejo, 2005). For genome size information, the Plant DNA C-values Database (Bennett & Leitch, 2010) and GSAD, a genome size database in the Asteraceae (Garnatje & al., 2010) were the main sources of information.

Statistical analyses

We analyzed statistical differences in genomic traits between widespread vs. mountain specialized plants, endemic vs. non-endemic for each plant community, and between both studied communities using the non-parametric Wilcoxon test (Quinn & Keough, 2002). All analyses were performed with JMP 9.0 software.

RESULTS AND DISCUSSION

The interest to study genome size and its significance has been increasing in recent years, mostly due to the emergence of flow cytometry (Loureiro & al., 2008), with many studies having used this character not only as a taxonomic marker, but also to evaluate how it correlates with phenotypic, ecological and environmental variables (e.g., Grime & Mowforth, 1982; Knight & Ackerly, 2002; Beaulieu & al., 2007; Knight & Beaulieu, 2008; for a review see Greilhuber & Leitch, 2013). So far, only a few studies have focused in studying this character at a natural community level, with the study of genome size variation in endemic genera of Macaronesia being one of the few exceptions of such an approach (Suda & al., 2003; Suda & al., 2005). Regarding the variation in chromosome numbers, due to the large amount of data obtained using classical karyology over the last 70 years, and the readability of the information in several web databases (e.g., Index of Plant Chromosome Numbers, Goldblatt & Johnson, 1976-) it is easier to find studies evaluating the incidence of polyploidy in particular communities, as the alpine flora (e.g., Nie & al., 2005). Still, most of those studies are based in bibliographic reviews, only. In here, besides an extensive review of the literature, we explored the variation in genome size and ploidy level of entomophilous taxa from two natural communities of high altitude.

Genome size variation

In the present work, the genome size of a total number of 191 individuals from 39 taxa was estimated (Table 1). From these, 32 (86.5%) constitute first estimations of genome size. With the exception of a few taxa (*Glandora diffusa*, *Reseda complicata*, *Scilla verna*, *Scorzoneroides microcephala*, *Silene rupestris* and *Viola riviniana*) the coefficient of variation (CV) of G_1 peaks were below 5% (Fig. 1). In these taxa, the high amounts of cytosolic compounds did not enable to achieve such CV values, and thus a higher CV threshold was considered acceptable (8%).

Among the studied species, a genome size variation of 39.2-fold was found (Fig. 2A), with the lowest mean value being obtained for *Pritzelago alpina* (2C = 0.36 ± 0.01 pg), whereas the highest one was obtained for *Scilla verna* (2C = 14.11 ± 0.27 pg). Regardless of this variation, according to Leitch & al. (1998) most of the estimations (56.4 %) fell in the very small genome category (2C < 2.8 pg), whereas 25.6% and 17.9% of the taxa presented small (2.8 pg < 2C ≤ 7.0 pg) and intermediate genome sizes (7.0 pg < 2C ≤ 28.0 pg), respectively. No species with large and very large genome sizes were detected. The overall genome size variation observed in the sampled taxa fits well with the distribution of C-values in angiosperms (Leitch & Leitch, 2013), which despite presenting a much larger range of variation (2342-fold for 6287 angiosperms), it is also strongly skewed towards very small and small genome sizes.

Most of the families to which the sampled taxa belong are well represented in the plant DNA C-values database (Bennett & Leitch, 2010). Nevertheless, for five families, there is a poor representation at the genus or species level, with estimations of genome size being available for 1-3 taxa only. Thus,

Table 1. Genome size and ploidy level in the studied high mountain taxa. The basic chromosome number (*x*), chromosome number (2n) and supposed DNA ploidy level of each taxon are given; when more than one value is given for basic chromosome number, chromosome number or ploidy level it reflects the impossibility to assign the obtained genome size to any ploidy level due to lack of previous genome size estimations for the taxon or even for the genus. Genome size values are given as mean, standard deviation of the mean and coefficient of variation of the holoploid genome size (2C, pg) of individuals of each taxon. The reference standard used to estimate the genome size (R.s.), the number of individuals analysed for genome size (*n* G.s.) and the total number of analysed individuals (*n* total) are also given. Also, for each taxon, previous genome size estimations, original references [(A)Lysak & al. (2009), (B)Boscaiu & al. (1999), (C)García-Fernández & al. (2012), (D)Marie & Brown (1993), (E)Bennett & Smith (1976), (F)Cires & al. (2009), (G)Cires & al. (2011)], herbarium vouchers availability and distribution information in the context of this work (mountain plant, and restricted endemism, R.e.) are provided. SN, Sierra Nevada; PE, Picos de Europa. R.s., *Raphanus sativus* 'Saxa' (2C = 1.11 pg); S.l., *Solanum lycopersicum* 'Stupické' (2C = 1.96 pg); P.s., *Pisum sativum* 'Ctirad' (2C = 9.09 pg); V.f., *Vicia faba* 'Inovec' (2C = 26.90 pg).

Montain range	Family	Species	DNA ploidy level			Genome size (pg/2C)			R.s.	Previous estimations	*n* G.s.	*n* total	H.v.	Mountain	R.e.
			x	2n	Ploidy level	Mean	SD	CV (%)							
SN	Asteraceae	*Carduus carlinoides* Gouan subsp. *hispanicus* (Kazmi) Franco	9	18	2*x*	2.30	0.06	2.7	S.l.	—	5	50	—	Y	Y
PE	Asteraceae	*Crepis pygmaea* L.	4	8, 12	2*x*, 3*x*, 4*x*	5.81	0.12	2.0	S.l.	—	5	5	RT37	Y	N
PE	Asteraceae	*Hieracium* gr. *mixtum*	—	—	—	10.23	0.33	3.2	S.l.	—	5	5	RT45	—	—
SN	Asteraceae	*Scorzoneroides microcephala* (Boiss.) Holub	6	12	2*x*	3.94	0.11	2.8	P.s.	—	9	9	RT17	Y	Y
PE	Boraginaceae	*Glandora diffusa* (Lag.) D.C.Thomas	8	16, 24, 32	2*x*, 3*x*, 4*x*	2.11	0.08	3.6	P.s.	—	5	5	RT36	N	N
PE	Brassicaceae	*Erysimum duriaei* Boiss.	7	14, 22, 26	4*x*	0.94	0.01	1.1	S.l.	—	3	3	RT38	Y	Y
PE	Brassicaceae	*Pritzelago alpine* Kuntze	6	12	2*x*	0.36	0.01	2.1	R.s.	0.38 (2*x*)(A)	5	5	RT42	Y	N
SN	Campanulaceae	*Campanula herminii* Hoffmanns. & Link	16	32	2*x*	2.86	0.06	2.2	S.l.	—	5	31	RT03	Y	N
SN	Campanulaceae	*Jasione crispa* (Pourr.) Samp. subsp. *tristis* (Bory) G.López	9	36	4*x*	8.93	0.12	1.4	S.l.	—	5	40	RT11	Y	Y
SN	Caryophillaceae	*Arenaria tetraquetra* L. subsp. *amabilis* (Bory) Laínz	20	40	2*x*	1.29	0.01	0.5	S.l.	—	5	32	RT08	Y	Y
PE	Caryophillaceae	*Cerastium arvense* L.	18	72	4*x*	2.81	0.02	0.5	R.s.	1.36 (2x), 2.6 (4x)(B)	5	5	RT43	N	N
SN	Caryophillaceae	*Cerastium cerastoides* (L.) Britton	18	36	2*x*	1.40	0.03	2.0	S.l.	—	5	49	RT16	Y	N
PE	Caryophillaceae	*Minuartia verna* (L.) Hiern	12	24, 48	2*x*, 4*x*	2.54	0.02	0.9	S.l.	—	4	4	RT51	N	N
PE	Caryophillaceae	*Minuartia villarsii* (Balb.) Wilczek & Chenevard	13	26	2*x*	2.30	0.05	2.3	S.l.	—	3	3	RT53	Y	Y
PE	Caryophillaceae	*Paronychia kapela* (Hacq.) A.Kern subsp. *serpyllifolia* (Chaix) Graebn.	9	18	2*x*	1.25	—	—	S.l.	—	1	1	—	Y	N
PE	Caryophillaceae	*Paronychia kapela* (Hacq.) A.Kern subsp. *serpyllifolia* (Chaix) Graebn.	9	36	4*x*	2.35	0.05	2.1	S.l.	—	4	4	RT46	Y	N
PE	Caryophillaceae	*Silene ciliate* Pourr.	12	24	2*x*	1.89	0.06	2.9	R.s.	1.73-1.82 (2*x*)(C)	4	4	RT56	Y	N
SN	Caryophillaceae	*Silene rupestris* L.	12	24	2*x*	3.40	0.08	2.4	P.s.	—	5	20	RT10	Y	N
SN	Caryophillaceae	*Spergularia rubra* (L.) J.Presl & C.Presl	9	18, 36, 54	2*x*, 4*x*, 6*x*	1.09	0.02	1.6	S.l.	—	5	50	RT09	N	N
PE	Crassulaceae	*Sedum atratum* L.	9	18, 36	2*x*, 4*x*	1.34	0.03	2.3	S.l.	—	5	5	RT34	Y	N
SN	Crassulaceae	*Sedum melanantherum* DC.	13	26	2*x*	0.64	0.01	1.9	S.l.	—	4	4	RT19	Y	N
PE	Fabaceae	*Hippocrepis comosa* L.	7	14, 28, 42	2*x*, 4*x*, 6*x*	5.88	0.09	1.5	S.l.	—	5	5	RT35	N	N
PE	Fabaceae	*Lotus corniculatus* L. subsp. *corniculatus*	6	18	3*x*	1.64	0.02	1.2	S.l.	0.90-1.40 (2x)(D), 2.10 (4x)(E)	5	5	RT40	Y	N
SN	Fabaceae	*Lotus corniculatus* L. subsp. *glacialis* (Boiss.) Valdés	6	12	2*x*	1.17	0.03	2.4	S.l.	0.90-1.40 (2x)(D), 2.10 (4x)(E)	5	50	RT07	Y	Y
SN	Lamiaceae	*Sideritis glacialis* Boiss.	17	34	2*x*	1.96	0.03	1.7	P.s.	—	5	50	RT20	Y	Y
PE	Lamiaceae	*Sideritis hyssopifolia* L.	16, 17	28, 30, 32, 34	2*x*	1.96	0.02	1.2	R.s.	—	3	3	RT47	N	N

Table 1. (Continuation).

Montain range	Family	Species	DNA ploidy level			Genome size (pg/2C)			R.s.	Previous estimations	*n* G.s.	*n* total	H.v.	Mountain	R.e.
			x	2n	Ploidy level	Mean	SD	CV (%)							
PE	Liliaceae	*Scilla verna* Huds.	10, 11	20, 22	2*x*	14.11	0.28	2.0	V.f.	—	5	5	RT41	N	N
SN	Plumbaginaceae	*Armeria splendens* (Lag. & Rodr.) Webb	9	18	2*x*	9.39	0.20	2.1	V.f.	—	5	25	RT18	Y	Y
SN	Ranunculaceae	*Ranunculus acetosellifolius* Boiss.	8	16	2*x*	8.97	0.05	0.5	S.l.	—	5	49	RT14	Y	Y
SN	Ranunculaceae	*Ranunculus demissus* DC.	8	16	2*x*	10.73	0.05	0.5	P.s.	—	5	50	RT05	Y	N
PE	Ranunculaceae	*Ranunculus parnassifolius* L. subsp. *heterocarpus* P.Küpfer	8	16	2*x*	7.37	0.07	1.0	P.s.	7.43-7.63 (2*x*), 15.09-15.85(4*x*), 20.45 (5*x*)[f]	5	5	RT50	Y	N
SN	Resedaceae	*Reseda complicata* Bory	14	28	2*x*	1.36	0.03	2.6	S.l.	—	5	50	RT23	Y	Y
PE	Rosaceae	*Alchemilla plicata* Buser	7	—	—	3.69	0.03	0.9	R.s.	—	5	5	RT39	Y	N
PE	Santalaceae	*Thesium pyrenaicum* Pourr.	7, 8	14, 16	2*x*	0.52	0.01	1.1	S.l.	—	3	3	RT52	N	N
PE	Saxifragaceae	*Saxifraga conifer* Coss. & Durieu	14	42	6*x*	1.31	0.02	1.9	S.l.	—	8	8	RT48	Y	Y
PE	Saxifragaceae	*Saxifraga hirsuta* L. subsp. *paucicrenata* (Leresche ex Gillot) D.A.Webb	14	28	2*x*	3.21	0.06	1.9	R.s.	—	5	5	RT49	Y	Y
PE	Saxifragaceae	*Saxifraga oppositifolia* L. subsp. *oppositifolia* L.	14	28, 52	2*x*, 4*x*	2.93	0.06	1.9	S.l.	—	5	5	RT55	Y	N
PE	Saxifragaceae	*Saxifraga paniculata* Mill.	14	28	2*x*	3.20	0.04	1.3	R.s.	—	5	5	RT44	Y	N
SN	Scrophulariaceae	*Euphrasia willkommii* Freyn	11	44	4*x*	1.70	0.03	1.6	S.l.	—	5	50	RT04	Y	N
PE	Violaceae	*Viola riviniana* Rchb.	10	20	2*x*	1.13	0.03	3.0	S.l.	2.55-2.97 (4*x*)[c]	5	5	RT54	N	N

our estimations for the two species of *Sideritis* (1.96 pg/2C) constitute the lowest values so far for this family (Lamiaceae, 2.88-12.30 pg/2C). By contrast, the estimation obtained for *Reseda complicata* (1.36 pg/2C), is the highest for Resedaceae (0.70-1.02 pg/2C). The values obtained for *Thesium pyrenaicum*, *Viola riviniana* and *Saxifraga* spp. (0.52; 1.13; 3.21 and 2.93 pg/2C, respectively) are within the range already observed in the few estimations of Santalaceae (0.58-0.62 pg/2C), Violaceae (2.61-2.70 pg/2C) and Saxifragaceae (1.35-4.76 pg/2C), respectively.

For the seven taxa whose genome size had been estimated before, a good agreement with previous results was observed, with the exception of *Lotus corniculatus* and *Viola riviniana*. Regarding *L. corniculatus*, we studied two different subspecies: for *L. corniculatus* subsp. *glacialis*, our estimation fits well in the variation reported for the diploids of this species (Marie & Brown, 1993), whereas for *L. corniculatus* subsp. *corniculatus*, considering the reported values for diploids and tetraploids of this species (Bennett & Smith, 1976), our estimations fall in the middle of the variation reported so far, suggesting the presence of DNA triploid individuals with 18 chromosomes; still as no infra-specific categorization was given in those works, this assumption should be confirmed in the future using classical karyology. For *V. riviniana*, despite of the difficulty to obtain histograms with low CV values, our estimation is approximately half that observed before for tetraploid individuals (Cires & al., 2011), which suggests that the sampled individuals are diploid with 20 chromosomes. Despite diploids have never been reported for this species, there are previous evidence of some variability in the number of chromosomes (2n = 35, 40, 45, 46, 47 chromosomes; Cires & al., 2011).

As reported before for several genera (e.g., *Helleborus* spp. and *Carthamus* spp., Zonneveld, 2001; Garnatje & al., 2006, respectively) and further confirmed in here (e.g., *Minuartia* spp., *Ranunculus* spp.) genome size can be an important extra taxonomic character for separating species, and potentially in the case of homoploids. In our work, when taxa of the same genus were analyzed, in most of the cases, it was possible to distinguish them using the genome size estimations, even when the supposed number of chromosomes was the same (e.g., *Ranunculus* spp., all with 16 chromosomes). This information can be particularly important for the identification of the two analyzed species of *Minuartia*, as they are very difficult to distinguish in the field. Still in the case of *Sideritis glacialis* and *S. hyssopifolia* and in the two taxa of *Saxifraga* with the same chromosome number (*S. hirsuta* subsp. *paucicrenata* and *S. paniculata*), the genome size was very similar, and thus, a not so useful character to distinguish those taxa. Considering the reported variability in chromosome numbers in *S. hyssopifolia* and its lack in *S. glacialis*, our results suggest for the occurrence of the same number of chromosomes in both species, i.e., 2n = 2*x* = 34. In all species, except *Hieracium* gr. *mixtum* and *Glandora diffusa*, a CV of genome size estimations below 3 % was obtained, suggesting the absence of intraspecific variation in genome size in most of the taxa.

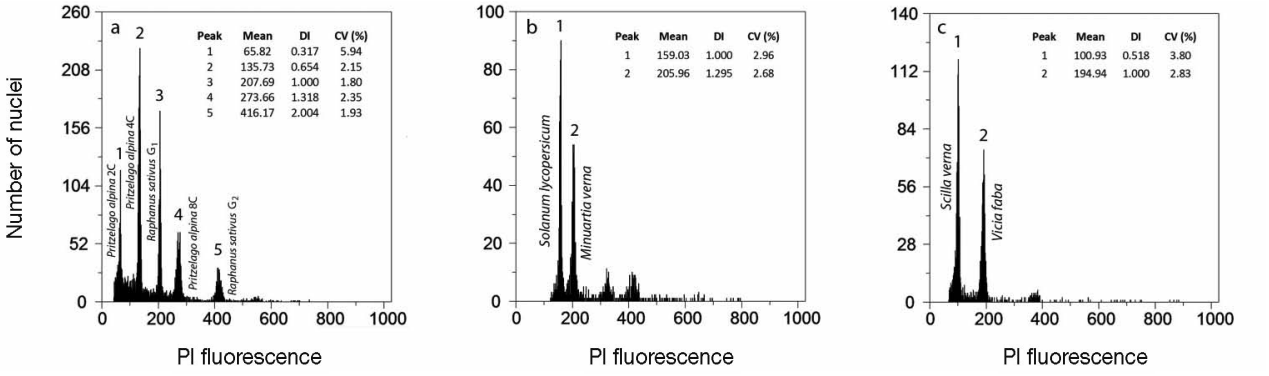

Fig. 1. Illustrative flow cytometric histograms of relative PI fluorescence intensity obtained after simultaneous analysis of nuclei isolated from the sampled taxa and the internal reference standard: (**a**) *Pritzelago alpina* with the occurrence of endoreduplication, and *Raphanus sativus*; (**b**) *Minuartia verna* and *Solanum lycopersicum*; (**c**) *Scilla verna* and *Vicia faba*. In each histogram, information of mean channel number (mean), DNA index (DI, defined as the ratio between the mean channel number of sample and reference standard) and coefficients of variation (CV, %) of each DNA peak are given. Please note the overall good quality of the histograms, as defined by the CV of each peak and by the low amount of background debris.

Polyploidy incidence

The DNA ploidy level of extra 526 individuals was estimated using the pooled sample strategy. For 30 out of 39 taxa, it was possible to infer the DNA ploidy level (Table 1), either using the information available in the chromosome databases or by comparison with previous genome size estimates. Of these taxa, seven were unequivocally polyploid: one potential triploid (*Lotus corniculatus* subsp. *corniculatus*), five tetraploids (*Cerastium arvense*, *Erysimum duriaei*, *Euphrasia willkommii*, *Jasione crispa* subsp. *tristis*, *Paronychia kapela* subsp. *serpyllifolia*) and one hexaploid (*Saxifraga conifera*), representing a polyploidy incidence of 23.3%, irrespective of the mountain range.

Polyploidy is thought to influence the capacity to tolerate environmental stress (Fawcett & Van de Peer, 2010) and may have granted some plant species a higher capacity to colonize new habitats after glaciation (Brochmann & al., 2004). Altogether, these beneficial traits have led to the assumption that polyploids are more frequent at higher latitude or altitude than related diploids (Löve & Löve, 1949; Love & Love, 1967). Indeed, some of these assumptions were confirmed recently when Brochmann & al. (2004) analyzed the incidence of polyploidy in the circumarctic flora. The authors observed that 60.7% of the artic plants are polyploids, with the frequency and level of polyploidy strongly increasing northwards within the Arctic. The evolutionary success of polyploids might be related with their fixed-heterozygous genomes, which buffered against inbreeding and genetic drift through periods of dramatic climate change. Regarding the incidence of polyploidy in alpine regions, only a few studies have addressed this issue. Löve & Löve (1967) observed a significantly high rate of polyploids in the alpine zone of the Mt. Washington (63.6%); also, Morton (1993) revealed an incidence of 52.9% in the flora of the Cameroon Mountains. By contrast, in the flora of the Hengduan Mountains, only 22% of the analyzed taxa were polyploids (Nie & al., 2005). Recently, Vamosi & McEwen (2012), observed that in the British Columbia flora (Canada) polyploids (especially those of hybrid origin) are disproportionately present at high elevation; still, no strong evidence that polyploids were tolerant of extreme or more varied environments than their diploid progenitors was found. The authors also found that mostly hybrids, some of which allopolyploids, had an increased elevational range, suggesting that the production of novel phenotypes, as well as, a wider range of allelic diversity, rather than masking of deleterious mutations, are a more important factor determining range limits of species. In our case, independently of the mountain range, an incidence of polyploidy of 23% was observed, which may be a first indication that this phenomoneon may have not been as important in shaping species adaptation to the alpine areas in these two distinct Spanish mountain ranges, as in other areas with extreme environment. Still, it was interesting to notice that even with such small dataset, five out of the seven polyploid taxa that were sampled are geographically restricted to high mountain areas.

Also, considering that polyploidization has long been recognized as a rapid mechanism of sympatric speciation (Adams & Wendel, 2005), it could help to explain rapid diversification and high endemism in a given region with high biodiversity. In some way, this contrasts with the idea that polyploids are generally considered to have a more widespread distribution than their related diploids, as a result of a higher capacity (due to successive hybridizations among differentiated polyploid populations) to colonize new environments after the retreat of Quaternary glaciers (Hodgson, 1987; Petit & Thompson, 1999). Indeed, as referred above, in the Hengduan Mountains polyploidy may have played a minor role, only, with geographical and ecological heterogeneity being considered to have played a more important role in the diversification of the plants of this region (Nie & al., 2005). Also, Petit & Thompson (1999) in the flora of the Pyrenees evaluated the relation between ploidy level, species diversity and ecological range, and observed that ploidy level had significant effects on the taxonomic diversity of the 50 genera studied, but not directly in the ecological range of genera. In Macaronesia, Atlantic islands with a complex and diverse flora and a high incidence of endemism, a very low incidence of polyploidy was repeatedly reported, with only 26.6% of the endemics and 27.8% of the total plants being polyploids (Borgen, 1974).

Within each taxon, we only found variation in ploidy level

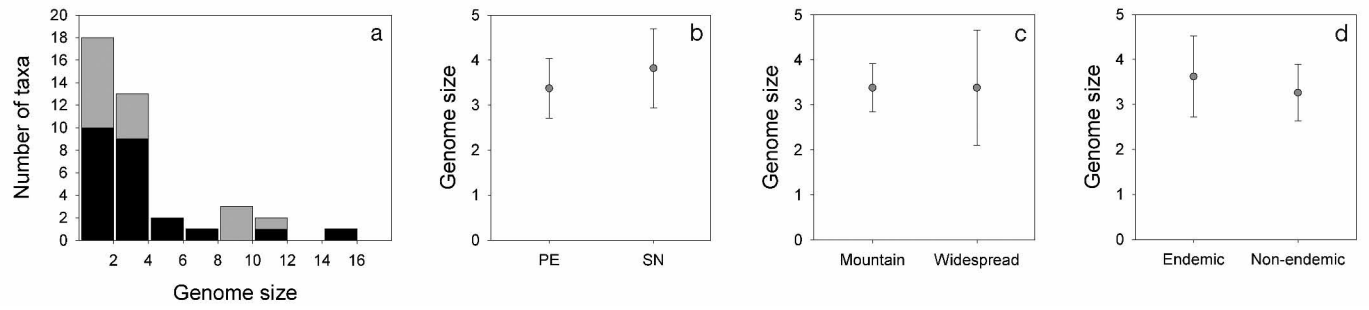

Fig. 2. Distribution of the genome size estimations among 39 high mountain taxa (**a**; black bars: Picos de Europa; grey bars: Sierra Nevada and comparisons of genome size variation (mean and SD, 2C/pg) between Picos de Europa (PE) and Sierra Nevada (SN) taxa (**b**), between plants specialized to live in mountain areas and those widely distributed (**c**), and between taxa endemic to restricted mountain ranges (Endemic) and not restricted (Non-endemic) (**d**).

once, with diploid and tetraploid individuals of *Paronychia kapela* subsp. *serpyllifolia* being detected in Picos de Europa. Both ploidy levels have already been reported for this subspecies, but no reference has been made regarding their occurrence in the same population (Küpfer, 1974). As shown before for other taxa (e.g., *Aster amellus*, Castro & al., 2012), contact zones of different cytotypes are very important as they constitute natural laboratories for studying evolutionary transitions in flowering plants (Lexer & van Loo, 2006). Therefore, future large and fine scale studies of cytotype distribution are necessary to improve the understanding of the evolutionary dynamics of the contact zone between diploids and tetraploids of *P. kapella* subsp. *serpyllifolia*.

Genome size, polyploidy, geographic origin and distribution ranges

The surveyed communities did not show statistical differences in genome size (Wilcoxon Test: $x_1 = 0.019$, $P = 0.890$, Fig. 2B). Genome sizes from Picos de Europa plants ranged from 0.36 ± 0.01 (*Pritzelago alpina*) to 14.11 ± 0.27 pg/2C (*Scilla verna*); whereas plants from Sierra Nevada ranged from 0.64 ± 0.01 pg (*Sedum melanantherum*) to 10.73 ± 0.05 pg/2C (*Ranunculus demissus*). Mountain specialists did not show significantly different genome sizes from those more widely distributed (Wilcoxon Test: $x_1 = 0.299$, $P = 0.584$, Fig. 2C). Furthermore, 13 of the species were narrow endemics and were mainly restricted to Sierra Nevada (Table 1). However, these endemic plants did not show different genome size than non-endemic ones (Wilcoxon Test: $x_1 = 0.044$, $P = 0.835$, Fig. 2D).

Regarding polyploidy, a higher frequency of polyploid taxa was observed in Picos de Europa than in Sierra Nevada (33.3% vs. 13.3%). Also, it is interesting to notice that most taxa inferred as polyploids were high altitude plants, except for *Cerastium arvense* and *Lotus corniculatus* subsp. *corniculatus* that can also grow in lowlands. The evaluation of polyploidy in endemic vs. non endemic plants, revealed a similar frequency of polyploids (23%); still, the two unique endemic species to the Cantabrian Range sampled in this study were polyploid (*Erysimum duriaei* and *Saxifraga conifera*). By contrast, from the eight sampled taxa endemic to Sierra Nevada, only one, *Jasione crispa* subsp. *tristis*, was polyploid, which reflects a percentage similar to the global incidence observed for this mountain area (12.5%).

Recent phylogenetic studies have evidenced multiple diversification patterns of alpine plants (Vargas, 2003). In the particular case of the two high mountain regions studied in here, there are already some examples where colonization and differentiation occurred from southeast Iberian Mountains to the Pyrenees (e.g., *Saxifraga pentadactylis* Lapeyr., *Arenaria tetraquetra* L.; Vargas, 2003). In the case of *A. tetraquetra*, the colonization and differentiation was accompanied with an increasing number of chromosome complements, with diploids being found in Sierra Nevada (subsp. *amabilis*). Still, molecular data in other plant groups also revealed that, in contrast to northern European areas where large-scale migrations occurred to recolonize territories after glacial periods, species in southern regions survived and diverged without large geographical displacements. The observed species range shift mostly involved populations ascending or descending mountains (Feliner, 2011). In our case, considering the differences between both mountain ranges in polyploidy incidence, it seems that, at least for the analyzed species of Sierra Nevada, either polyploidy was not involved in the short-distance expansion occurred after glaciation, or, as hypothesized for *Armeria splendens* (Larena & al., 2002), those taxa constitute the first inhabitants in Sierra Nevada, which despite the events of gene flow and hybridization with taxa from lower altitude during climatic changes in the Pleistocene (eventually leading to the formation of polyploid taxa that subsequently expanded to other areas), survived and lasted in high altitude refugia until the present time.

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