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ORIGINAL ARTICLE

Genome size variation and morphological differentiation within *Ranunculus parnassifolius* group (Ranunculaceae) from calcareous screes in the Northwest of Spain

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Abstract *Ranunculus parnassifolius* is an orophilous plant distributed throughout Central and Southwestern Europe (Alps, Pyrenees and Cantabrian Mountains). Its evolutionary history and taxonomy are often complicated, having been little studied before now. The purpose of this article is to present flow cytometry measurements and multivariate morphometric analyses to ascertain cytotype distribution patterns and the morphological differentiation of *R. parnassifolius s.l.* from calcareous screes in the Northwest of Spain. DNA ploidy level and morphometric analysis were determined for plants of *R. parnassifolius s.l.* using flow cytometry (112 individuals) and multivariate analysis (152 individuals). Specimens were collected in eight localities in the Northwest of the Iberian Peninsula. Different sample preservation methods (fresh, frozen, and herbarium specimens) were employed as well as the use of various buffers and internal standards, in order to test the reproducibility of DNA flow cytometry. Three ploidy levels were detected in the study area (diploid, tetraploid, and pentaploid), and mixed-cytotype populations were also found. The mean nuclear DNA content of the *R. parnassifolius* group ranged from 7.43 ± 0.185 to 7.63 ± 0.339 pg/2C in diploids and from 15.09 ± 0.161 to 15.85 ± 0.587 pg/2C in tetraploids. The analysis of the monoploid genome sizes (1Cx) did not reveal a clear difference among cytotypes. These results suggest low intraspecific variation, at least among the populations studied. In addition, a comparison of different DNA reference standards was conducted. A new value for the chicken genome size was used as internal reference standard ($2C = 3.14 \pm 0.155$ pg), with similar results found using both animal and plant standards (*Pisum sativum* and *Solanum lycopersicum*). Finally, herbarium vouchers and frozen tissue were proved to be suitable for DNA ploidy level measurements. This study provided a first assessment of C values in the *R. parnassifolius* group using flow cytometry. The weak morphological distinction of the cytotypes and the existence of mixed-cytotype populations in the Northwest of Spain are reported here for the first time. The different distribution pattern of the two cytotypes is discussed.

Keywords Flow cytometry · Genome size · Iberian Peninsula · Multivariate morphometric analysis · Ploidy levels · *Ranunculus parnassifolius*

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Introduction

Ranunculus L. represents the largest genus within Ranunculaceae and comprises nearly 600 species (Tamura 1993), distributed from temperate to artic/subantarctic zones (Ziman and Keener 1989), being rare in the tropics where it is restricted to high mountain areas (Hörandl et al. 2005). According to the systematics proposed by Cook et al. (1986), in the Iberian Peninsula around 65 species are recognized, a quarter of which are endemic. They are divided into three subgenera: *Ranunculus* subgen. *Batrachium* (DC.) A. Gray (8 species), *Ranunculus* subgen. *Ficaria* (Schaeff.) L. Benson (1 species), and *Ranunculus* subgen. *Ranunculus*

L. This latter subgenus, *Ranunculus*, includes at least 56 species classified into 13 sections. One of them, the section *Ranuncella* (Spach) Freyn, comprises a group of plants spread throughout Central and Southwestern Europe and with a local presence in the North African Mountains (*Ranunculus calandrinioides* Oliv.) and in the Korab Mountains in the Republic of Macedonia (*R. wettsteinii* Dörfl.) (Jalas and Suominen 1989; Tutin and Akeroyd 1993). In the Iberian Peninsula, seven species from this section *Ranuncella* are represented (*Ranunculus abnormis* Cutanda & Willk., *R. amplexicaulis* L., *R. angustifolius* DC., *R. bupleuroides* Brot., *R. gramineus* L., *R. parnassifolius* L., and *R. pyrenaeus* L.), most of them being endemic to small areas.

Traditionally, *R. parnassifolius* has been considered an orophilous plant distributed through the Alps, the Pyrenees, and the Cantabrian Mountains (Tutin 1964); nevertheless, some authors studying distribution in different locations have attributed different taxonomic ranks (from varietal to species level) to the taxa *R. parnassifolius* (Candolle 1817; Rothmaler 1934; Guinea López 1953). After obtaining data from across the range of *R. parnassifolius s.l.*, Küpfer (1974) proposed the following systematics, which has been accepted by most authors (Cook et al. 1986; Jalas and Suominen 1989; Tutin and Akeroyd 1993): *R. parnassifolius* subsp. *parnassifolius* diploid ($2n = 16$), silicicole, endemic in the Eastern Pyrenees; *R. parnassifolius* subsp. *cabrerensis* Rothm. diploid ($2n = 16$), silicicole, endemic to the mountains of Northwestern Spain; *R. parnassifolius* subsp. *favargeri* P. Küpfer diploid ($2n = 16$), calcicole, endemic in the Picos de Europa (Cantabrian Mountains) and Western Pyrenees; and finally, *R. parnassifolius* subsp. *heterocarpus* P. Küpfer tetraploid ($2n = 32$, 40), calcicole, spread throughout the Cantabrian Mountains, Central Pyrenees, and Alps. Later, Bueno Sánchez et al. (1992) described another subspecies, *R. parnassifolius* subsp. *muniellensis* Bueno, Fern. Casado & Fern. Prieto, as diploid ($2n = 16$), silicicole, with a single population in the Biological Reserve of Muniellos (Western Cantabrian Mountains). Despite the relatively small area covered by the Cantabrian Mountains, it may be considered to be the area with the highest global diversity of *R. parnassifolius*, with four out of five of the recognized subspecies being found within it (Küpfer 1974; Cook et al. 1986; Bueno Sánchez et al. 1992).

Although many authors have reported the presence of *R. parnassifolius* subsp. *favargeri* and *R. parnassifolius* subsp. *heterocarpus* in the calcareous screes of the Cantabrian Mountains (e.g., Küpfer 1974; Laínz 1976; Fernández Prieto 1983; Rivas Martínez et al. 1984; Nava 1988), chromosome counts from these natural populations have only been reported by Küpfer (1969, 1974). In the cited studies, a chromosome count was undertaken on a total of three plants (two tetraploid and one diploid) in very close locations in the Central Massif of the Picos de Europa, this being the first and only record in the Cantabrian Mountains. In all other published work, identification has been solely based on morphological characters provided by Küpfer (1974), such as the relationship between the number of stamens and carpels, the presence of microspores and carpels aborted, and the regularity of the corolla. However, although ploidy level is considered a differentiating feature among these taxa, there is little information about the within- and among-population variation in ploidy levels in natural populations of limestone-dwelling *R. parnassifolius*.

Polyploidy is recognized as an important evolutionary phenomenon and a significant source of angiosperm diversity (Soltis et al. 2007), thus the geographical distributions of cytotypes, within species or between closely related species, provide useful insights into the evolutionary history of polyploid complexes (Kron et al. 2007; Perný et al. 2008; Ståhlberg and Hedrén 2008). Therefore, one aim of polyploidy research is to obtain better characterization of the distribution of cytotypes at various spatial and temporal scales. In recent years, a growing body of data has become available concerning the geographical distribution of cytotypes (e.g., Mandáková and Münzbergová 2008; Perný et al. 2008; Španiel et al. 2008). Nevertheless, even if the cytotypes represent distinct biological entities, present taxonomic treatments largely rely on a morphology-based concept, and assessment of their morphological differentiation is therefore still highly relevant (Soltis et al. 2007).

Flow cytometry (FCM) has become a frequently used method in the estimation of DNA ploidy and genome size in plants. The basic process of plant FCM is as follows: isolation of nuclei from plant material, staining of their DNA with fluorochrome, followed by analysis of their fluorescence emission. It is a convenient method that can provide useful information for phylogenetic and taxonomic purposes and is proving to be a useful tool in delimitation between some species that are sometimes not so easily distinguished using morphoanatomic analyses (e.g., Loureiro et al. 2007; Perný et al. 2008). However, it is widely accepted that FCM has some drawbacks. A common problem is related to the difficulty of the estimation of DNA content for various reasons, such as the isolation of insufficient intact nuclei for analysis, the aggregation of fluorescent debris particles to the surfaces of isolated nuclei, or the decreasing resolution of DNA content histograms. Considering that the estimation of DNA content in absolute units requires internal standardization and that it is necessary to have a close but non-overlapping genome size in relation to the target species, the use of different plant and animal standards in different studies has to be taken into account when comparing results. In short, some

striking discrepancies between flow cytometry estimations of genome sizes in different laboratories may be explained by the use of different reference standards, sample preparation and staining protocols, and flow cytometers (Doležel et al. 1998). In fact, these differences in peak ratios obtained in different laboratories play an important role in the correct estimation of plant genome size. Additionally, the buffer composition is crucial for obtaining precise, reliable, and high-resolution results; hence a first step in studying FCM is to test different buffers, selecting the most suitable. According to Loureiro et al. (2006b), the most appropriate buffer depends on the species, the tissue type, and the presence of cytosolic compounds that interfere with DNA staining.

Based on the current knowledge of the *R. parnassifolius* group in the Cantabrian Mountains, we addressed the following questions: (1) What is the frequency of the different cytotypes? (2) Are diploids and tetraploids morphologically differentiated? If so, which morphological characters contribute to their differentiation? (3) Are there any mixed-cytotype populations in the study area? and (4) What is the spatial distribution pattern of both cytotypes in this geographical area? In addition, it is hoped that this work will contribute to a more thorough knowledge of FCM in plant cells of the *R. parnassifolius* group of the Cantabrian Mountains, through the evaluation of different sample preservation methods (fresh, frozen, and herbarium vouchers), as well as the use of various buffers and internal standards for the estimation of nuclear DNA content in the studied populations and for testing the reproducibility of DNA flow cytometry for genome size estimation.

Materials and methods

Study area and plant material

The studied populations of *R. parnassifolius* are situated in the Cantabrian Mountains, located in the Northwest of Spain and running from east to west, parallel to the Atlantic coast (at a distance from the coast never exceeding 80 km) (Fig. 1a, b). The climate of the area is temperate and humid, and at higher levels, especially in the oro-template belt (subalpine zone), snow cover extends from the middle of autumn until at least early spring. In the northeastern area of these Cantabrian Mountains, dominated by paleozoic limestones, are the Picos de Europa, an area divided into three independent massifs: the Western Massif (El Cornión), the Central Massif (Los Urrieles), and the Eastern Massif (Ándara). All of them are almost totally calcareous, with peaks of around, or even over, 2,500 m. It should be noted that, although separated geographically from the Picos de Europa, Peña Ubiña and Picos Albos also form part of the Cantabrian Mountains. In all three areas, the rock type and the extreme climatic variations cause strong freeze–thaw processes, with resulting gravels and pebbles abundant in a wide extension along the mountainside.

Plant samples of eight populations of *R. parnassifolius s.l.* were collected; one population from Picos Albos (Somiedo Natural Park, Biosphere Reserve) and the other seven populations from the three massifs of the Picos de Europa (Picos de Europa National Park, Biosphere Reserve) (Table 1). Sampling was carried out during the summers of 2007 and 2008, between the months of May and July. Geographical coordinates were recorded in the field (Garmin-Etrex GPS instrument), and topographic information was derived from the geographic information system (GIS), the resulting data being processed with GIS software (ArcGis 9.2, ESRI). The identification of each plant was verified by consulting the original description of the species and other relevant literature. As a rule, 15–20 plants from each population were sampled. Efforts were made to avoid collecting samples originating from one clone (usually plants growing very close together in a very small area of several square centimeters). Field-collected specimens were potted and kept in a greenhouse at room temperature and are currently maintained as a collection of living plants at the Botany Area of the University of Oviedo. Leaves taken from plants at the site were wrapped in moistened paper enclosed in a plastic bag and transported in a cool box to avoid high temperatures during transportation; then frozen plant tissues were stored at −80°C. Additionally, voucher specimens were collected, dried by pressing in absorbent paper, stored at room temperature, and kept in the Herbarium of the University of Oviedo (FCO).

Genome size estimations using flow cytometry

An assessment of nuclear isolation buffers was previously carried out, comparing the most common buffers of different chemical compositions: LB01 [15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 15 mM of β-mercaptoethanol, 0.1% (v/v) Triton X-100, pH 7.5] (Doležel et al. 1989), Otto's [Otto I: 100 mM citric acid monohydrate, 0.5% (v/v) Tween 20 (pH approx. 2–3); Otto II: 400 mM $Na_2HPO_4 \cdot 12H_2O$ (pH approx. 8–9)] (Otto 1990; Doležel and Göhde 1995), and Tris.$MgCl_2$ [200 mM Tris, 4 mM $MgCl_2 \cdot 6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5] (Pfosser et al. 1995). In order to compare the performance of these nuclear isolation buffers, a set of parameters was selected to evaluate sample quality. Among the parameters chosen, the coefficient of variation of DNA peaks (CV = standard deviation/peak mean $\times$ 100%) was of major importance.

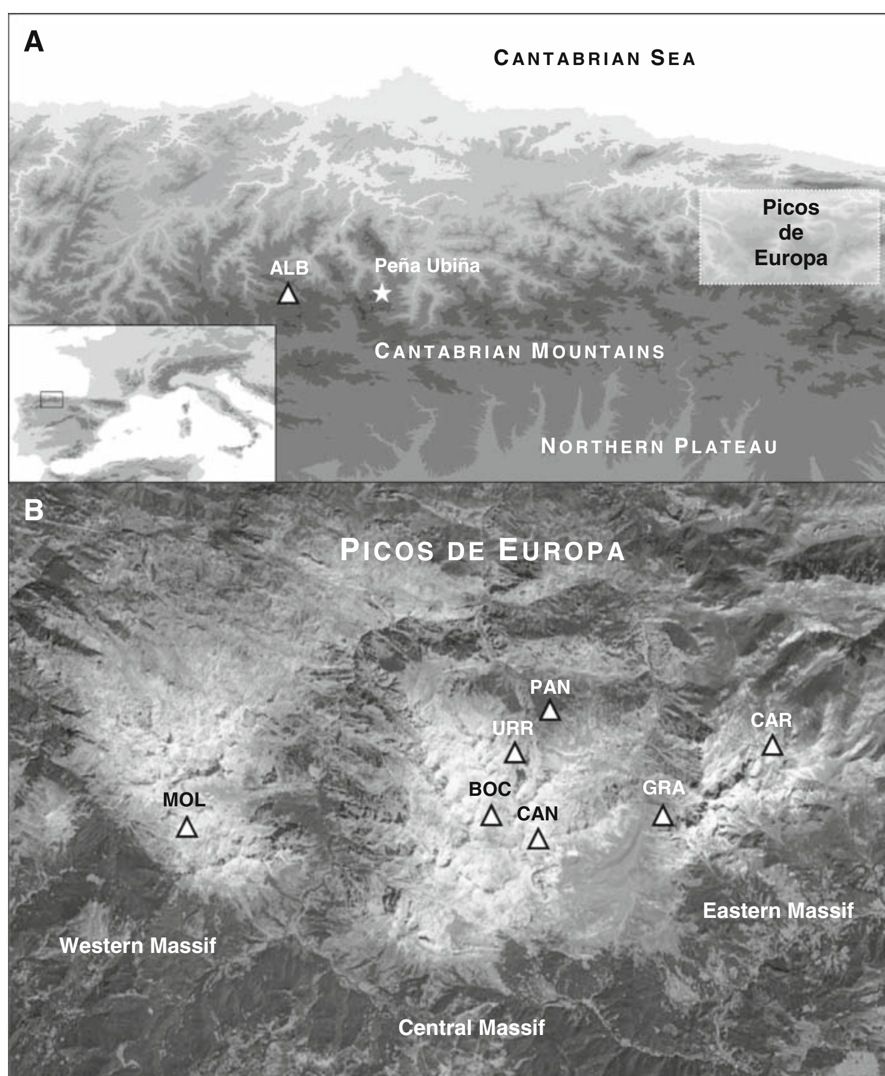

Fig. 1 Map of the study area. **a** Geographical distribution of *R. parnassifolius s.l.* in the Cantabrian Mountains, including the sampled area of Picos Albos (ALB). **b** Sampled territories in the Picos de Europa. *Triangles* refer to localities of sample collection (BOC, CAN, CAR, GRA, MOL, PAN, URR). Image modified from Google Earth 4.0

In analyzing each plant, the CV of the standard and the sample were calculated, considering 5.0% as the maximum acceptable CV value in plant DNA flow cytometry (Greilhuber et al. 2007). In order to check the presence of substances that interfere with the intercalating fluorescent dye, all the plant species used for the DNA content determination were tested by FCM; an inhibitor test was conducted (Greilhuber 2008), putting the samples one on top of another in a sandwich-like fashion and chopping into sections of both materials (standard and sample).

For FCM analyses, nuclear suspensions were prepared according to the protocol of Galbraith et al. (1983). A new razor blade was used to simultaneously chop 150 mg of leaf tissue of *R. parnassifolius s.l.* and 50 mg of internal standard leaves in a glass Petri dish containing 1 mL of ice-cold LB01 nuclear isolation buffer (the buffer having been stored frozen at −20°C in aliquots for convenient use). The samples were maintained at ice-cold temperature following isolation of nuclei in order to decrease nuclease activity. Then, the nuclear suspension was filtered through a 42 μm nylon filter to remove large debris, and 50 μg mL $^{-1}$ of propidium iodide (PI, Sigma) was added to the samples to stain the DNA. As propidium iodide is an intercalating fluorescent dye that binds to DNA and double-stranded RNA, samples were treated with RNase (50 μg mL $^{-1}$, Sigma) to avoid the staining of double-stranded RNA. After mixing well, the samples were put on ice and kept in darkness for a 30-min period and then analyzed. Experiments were carried out using a Cytomics FC 500 (Beckman Coulter) with 488-nm excitation from an argon ion laser. Data analysis was carried out using Cytomics RXP Analysis (Beckman Coulter).

At least three individuals from each population were analyzed on three different days to avoid errors due to instrumental drift, and at least 5,000 nuclei were analyzed per sample. The holoploid genome size (2C; sensu

Table 1 Collection locations of the *R. parnassifolius s.l.* samples

Acronym	Locality and collector	Zone	Coordinates	Altitude (m a.s.l.)	Date	Voucher specimens
ALB	Massif of Picos Albos, above Lago Cerveriz (Lagos de Saliencia, Somiedo, Asturias); *EC & JAFP*	29T	$x = 734{,}311$ $y = 4{,}770{,}131$	1,946	2007-06-18 2008-07-01	FCO: 31103; 31104
BOC	Central Massif of the Picos de Europa, Jou de los Boches (Cabrales, Asturias); *EC*	30T	$x = 351{,}469$ $y = 4{,}783{,}099$	2,136	2007-07-13 2008-07-10	FCO: 31105; 31106
CAN	Central Massif of the Picos de Europa, Collado de la Canalona (Camaleño, Cantabria); *EC*	30T	$x = 352{,}498$ $y = 4{,}782{,}342$	2,455	2007-07-11 2008-07-07	FCO: 31107; 31108; 31109; 31110
CAR	Eastern Massif of the Picos de Europa, Collado de San Carlos (Camaleño, Cantabria); *EC*	30T	$x = 362{,}152$ $y = 4{,}785{,}130$	2,050	2008-07-30	FCO: 31111; 31112
GRA	Eastern Massif of the Picos de Europa, Canal de las Grajas (Camaleño, Cantabria); *EC*	30T	$x = 357{,}333$ $y = 4{,}782{,}264$	1,688	2008-07-30	FCO: 31113
MOL	Western Massif of the Picos de Europa, Los Moledizos (Posada de Valdeón, León); *EC*	30T	$x = 340{,}430$ $y = 4{,}782{,}283$	2,043	2008-07-30	FCO: 31114
PAN	Central Massif of the Picos de Europa, pathway from Pandébano to Vega de Urriellu. Opposite Jou Lluengu (Cabrales, Asturias); *EC*	30T	$x = 352{,}622$ $y = 4{,}786{,}134$	1,632	2007-07-12 2008-05-20	FCO: 31115; 31116
URR	Central Massif of the Picos de Europa, Vega de Urriellu just past the refuge J.D. Ubeda in the direction of Horcados Rojos (Cabrales, Asturias); *EC*	30T	$x = 352{,}074$ $y = 4{,}785{,}006$	1,967	2007-07-12 2008-07-10	FCO: 31117; 31118; 31119

Geographical coordinates and altitudes are in accordance with European 1950 and Universal Transverse Mercator datums. Collectors: *EC*, E. Cires; *JAFP*, J.A. Fernández Prieto

Greilhuber et al. 2005) of *R. parnassifolius* subspecies was estimated according to the following formula:

$$\text{2C nuclear DNA content (pg)} = \frac{\textit{Ranunculus}\ \text{sp. G0/G1 peak mean}}{\text{reference standard G0/G1 peak mean}} \times \text{nuclear DNA content of reference standard}$$

The monoploid genome size (1Cx; sensu Greilhuber et al. 2005) of all plants was also calculated in mass values (pg) and Mbp (1 pg = 978 Mbp, Doležel et al. 2003).

Due to very low mitotic activity in *R. parnassifolius* leaf tissues (as indicated by the absence or low incidence of peaks corresponding to nuclei in G2 phase) and the lack of endopolyploidy, potential bias in DNA ploidy level estimation can be excluded.

DNA reference standards

Plant and animal standards were used for the present work. *Pisum sativum* cv. 'Ctirad' (2C = 9.09 pg of DNA, Doležel et al. 1998) and *Solanum lycopersicum* cv. 'Stupicke' (2C = 1.96 pg of DNA, Doležel et al. 1992), both of which have a genome size close to that of *R. parnassifolius s.l.* samples, were used as internal reference standards for all the studied species to minimize potential instrument non-linearity. Seeds from both cultivars were supplied by the Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany (Olomouc, Czech Republic), and germinated in a greenhouse at 22 ± 2°C. Additionally, nuclei from chicken red blood cells (CRBC; 2C = 2.33 pg of DNA, Galbraith et al. 1983), kept at the Flow Cytometry Area (Scientific-Technical Services, the University of Oviedo), were used for plant DNA flow cytometry analysis. Fixed CRBC were self-prepared and stored at low temperatures (J. Doležel, unpublished results; http://lmcc.ieb.cz/research/protocols.php?protocols=particles). Only one male domestic chicken (*Gallus gallus domesticus*) was used throughout the analysis, and the nuclei from the animal reference standard were added after preparing plant nuclei suspension.

Morphometric measurements

Materials from all localities (20 plants per population) were subjected to multivariate morphometric study. Characters measured or scored, always made on fresh specimens, are

listed in Table 2. The selection of the morphological characters to be scored followed a literature review of previous taxonomic studies on this species and a preliminary examination of herbarium material. They included characters traditionally used for differentiation of the two recognized taxa/cytotypes according to accepted determination keys and floras. For logistical reasons, it was not possible to collect samples of each population in exactly the same flowering and/or fructification period. In addition to this, in some specimens, it was not possible to measure all the characters because the specimen either did not contain enough flowering individuals or had been damaged by grazing. These facts should be taken into consideration in the interpretation of the morphometric analysis results.

Statistical analysis

Genome size data obtained by FCM and differences among standards were analyzed using Kruskal–Wallis one-way ANOVA on ranks, and Dunn's method for pair-wise comparison. For each species, within-population differences in nuclear DNA content and differences among sample preservations were analyzed (when applicable) and compared using a one-way ANOVA procedure, and a Holm–Sidak multiple comparison test was used for pair-wise comparison. Basic statistical measures for the morphometric analysis were evaluated using Mann–Whitney rank sum test. All statistical studies were performed using Statistica 7 (StatSoft).

Table 2 List of characters measured and used in morphometric analyses of *R. parnassifolius s.l.* samples

Character	Description
Continuous quantitative characters	
LL	Lamina length of the basal stem leaf
LW	Lamina width of the basal stem leaf
LS	Length of sepals
WS	Width of sepals
LP	Length of petals
WP	Width of petals
LA	Length of achene
WA	Width of achene
Discrete quantitative characters	
NF	Number of flowers
NS	Number of sepals
NP	Number of petals
Ratio characters	
LLLW	Lamina length of the basal stem leaf/lamina width of the basal stem leaf
LSWS	Length of sepals/width of sepals
LPWP	Length of petals/width of petals
LAWA	Length of achene/width of achene
Binary characters	
SL	Base shape of the basal leaves (0: ovate-subcordate; 1: broadly cordate)
IAD	Indumentum of adaxial surface of basal leaves (0: basal leaves uniformly hairy above; 1: basal leaves more densely hairy on the veins than elsewhere above or occasionally only at base)
IAB	Indumentum of abaxial surface of basal leaves (0: basal leaves hairy beneath; 1: basal leaves glabrous beneath, except sometimes at base)
TI	Type of inflorescence (0: dense cyme; 1: open cyme)
IS	Indumentum of sepals (0: sepals densely villous; 1: sepals glabrous or sparsely hairy)
FS	Floral regularity (0: corolla usually irregular, with unequal or absent petals; 1: corolla more or less regular)
CL	Color of petals (0: petals white; 1: petals usually pinkish white)
SA	Surface of achene (0: achenes strongly veined; 1: achenes smooth or with inconspicuous veins)
NSTC	Number of stamens/number of carpels (0: ≤ 1, 1: > 1)

In terms of morphometric measurement, exploratory data analysis was used for each DNA ploidy level to obtain basic statistics related to quantitative and ratio characters (mean, standard deviation, minimum and maximum values, 5th and 95th percentiles). The morphometric information based on quantitative, binary, and ratio characters was analyzed using Principal Coordinates Analysis (PCoA) and Principal Components Analysis (PCA), techniques commonly used in numerical classifications. These methods reduce the dimensions of the original data and allow visual interpretation of the relationships. The principal components of PCA are linear combinations of the original descriptors. In contrast, principal coordinates in PCoA are complex functions of the original descriptors mediated through a dissimilarity measure. Therefore, the relative position of the points is an indication of their taxonomic relationship. For the PCoA, a similarity matrix (S) was first built, using the Gower similarity coefficient (Gower 1971) that permits simultaneous work on qualitative and quantitative characters. Then, a dissimilarity (or distance) matrix (D) was built. Distance and similarity are intimately connected through a set of mathematical formulas, where $D = 1 - S$ (D distance; S similarity) is the simplest (Escudero et al. 1994). Based on this new matrix, a PCoA was carried out in order to display the "natural groups" that might appear in the sample and identify the factors that may be responsible for such clusters. Different PCoA and PCA were run, including all vegetative (LL, LW, LLLW, SL, IAD), floral (LS, WS, LP, WP, NF, NS, NP, LSWS, LPWP, TI, IS, FS, CL, NSTC) and/or fructification characters (LA, WA, LAWA, SA) (see Table 2). In addition, multivariate analysis methods were performed using Ginkgo version 1.5.8 software (Bouxin 2005), the statistical module from the VegAna package (http://biodiver.bio.ub.es/vegana), that provides several tools for editing and analyzing flora and vegetation.

Results

Nuclear DNA content estimations

The 2C nuclear DNA content of 112 specimens of *R. parnassifolius s.l.* was determined using FCM, providing histograms with well-defined peaks of both sample and internal reference standards (CRBC) (Table 3). Three different DNA ploidy levels were revealed in the study area: diploid ($2n \sim 2x$, 52.67%), tetraploid ($2n \sim 4x$, 44.64%), and pentaploid ($2n \sim 5x$, 2.67%). Mean holoploid genome size of diploid plants (2C) ranged from 7.43 pg/2C in URR to 7.63 pg/2C in BOC, whilst in tetraploid plants the holoploid genome size ranged from 15.09 to 15.85 pg/2C in CAR and ALB, respectively. Statistical analyses revealed significant differences between DNA ploidy level in all tested populations ($P < 0.001$). A high homogeneity of the values within each ploidy was also observed. The occurrence of pentaploid plants deserves particular interest as it represents the second recorded incidence in the

Table 3 Nuclear DNA content estimations of the *R. parnassifolius* group studied in this work

Population	Ploidy level	2C (pg)	2C range		Dif. pop.	1Cx (pg)	1Cx (Mbp)	CV (%)	*n*
			Min.	Max.					
ALB	4*x*	15.85 ± 0.587	14.76	16.81	a	3.96 ± 0.147	3,877	4.37	13
	5*x*	20.45 ± 0.975	19.32	21.04	b	4.09 ± 0.195	4,000	5.33	3
MOL	4*x*	15.30 ± 0.432	14.74	15.94	a	3.82 ± 0.108	3,743	4.52	10
PAN	2*x*	7.54 ± 0.145	7.24	7.74	c	3.77 ± 0.072	3,689	3.34	16
URR	2*x*	7.43 ± 0.185	7.19	7.75	c	3.71 ± 0.092	3,636	3.98	14
	4*x*	13.94	–	–	a	3.48	3,409	4.57	1
BOC	2*x*	7.63 ± 0.339	7.13	8.32	c	3.81 ± 0.170	3,734	4.64	15
CAN	2*x*	7.57 ± 0.471	7.08	8.53	c	3.78 ± 0.235	3,705	3.79	14
GRA	4*x*	15.25 ± 0.463	14.73	16.18	a	3.81 ± 0.116	3,729	3.27	10
CAR	4*x*	15.09 ± 0.161	14.79	15.31	a	3.77 ± 0.040	3,689	3.82	16

The values are given as mean and standard deviation of the holoploid nuclear DNA content (2C in pg) of individuals of each population. The 2C range is defined by the minimum (*Min.*) and maximum (*Max.*) value obtained for each population. The monoploid nuclear DNA content (*1Cx*) in mass values (pg) and Mbp, the mean sample coefficient of variation of G0/G1 DNA peak (*CV*, %) and the number of analyzed individuals (*n*) are also provided for each population

Differences among populations (*Dif. pop.*) were analyzed using Kruskal–Wallis one-way ANOVA on ranks and Dunn's method. Differences within populations were analyzed using a one-way ANOVA procedure and a Holm–Sidak multiple comparison test. Populations identified by the same letter are not statistically different ($P < 0.001$)

Internal reference standard: CRBC 2C = 3.14 pg of DNA (this study); 1 pg = 978 Mbp (Doležel et al. 2003)

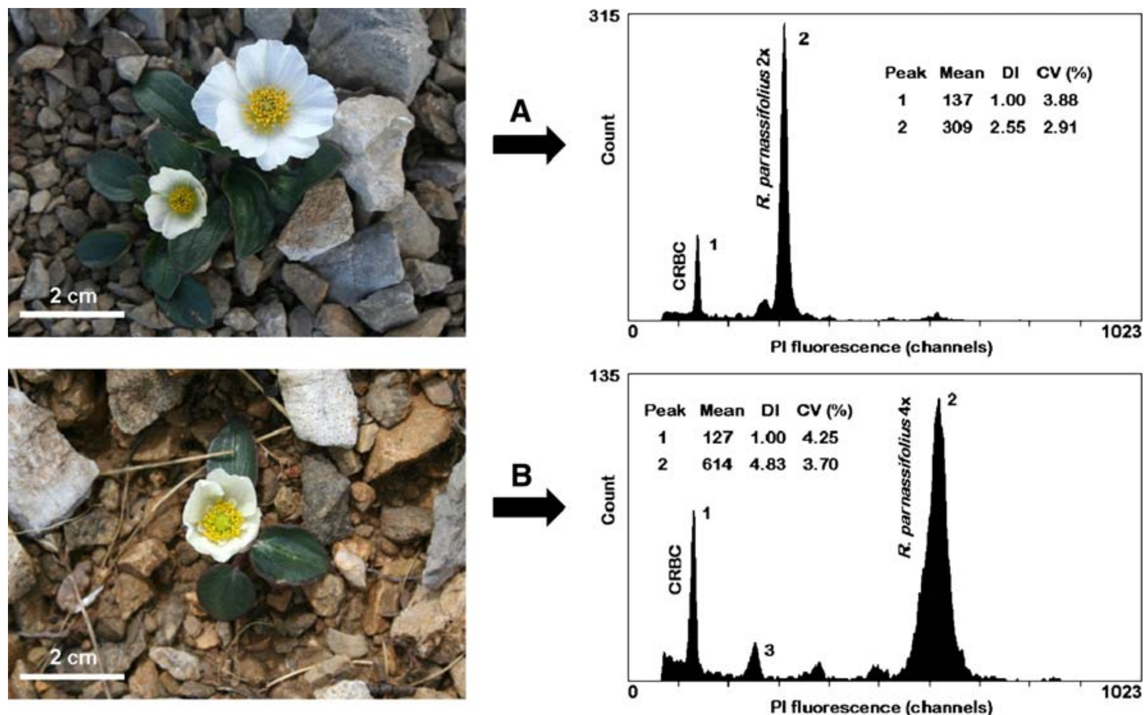

Fig. 2 Morphological aspect and histograms of relative fluorescence intensity obtained after analysis of propidium-iodide-stained nuclei of *R. parnassifolius s.l.* **a** Diploid plant from CAN, Central Massif of the Picos de Europa. **b** Tetraploid plant from ALB, Somiedo. The peaks marked with *1* and *2* indicate nuclei at the G0/G1 phase of the internal standard and the G0/G1 phase of the sample, respectively. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. In the histogram in **b**, the number *3* corresponds to G2/M peaks

species. Fluorescence histograms (Fig. 2a, b) of relative nuclear DNA content showed diploid and tetraploid plants with CVs usually below 4.0% (3.97 ± 0.778, mean ± SD; $n = 109$). Indeed, 88.07% of the estimations presented a CV value below 5.0%, and in only 11.93% of cases were these values above 5.0% (CV maximum value 5.50%). Intraspecific variation in genome size within each cytotype was detected (2.71% in diploid and 5.12% tetraploid), but on overall examination, the analysis of the monoploid genome sizes (1Cx) did not reveal a clear difference between diploid and tetraploid level (Table 3), the low variability thus presumably being due to instrumental or methodological errors.

Comparison of different DNA reference standards

For the estimation of the genome size in absolute units, an internal standard of "known" genome size was co-processed with the specimen. In this way, internal standardization eliminates the risk of error due to variations in sample preparation and instrument instability. In the resulting flow histogram, the ratio of specimen/standard peak means was calculated and was then multiplied by the absolute genome size of the reference standard to give the absolute genome size of the specimen. Therefore, as accurate a genome size of internal standard as possible should be used in order to compare the results with other works. The nuclear DNA content of *R. parnassifolius s.l.* samples was initially determined by assuming the value of CRBC nuclei as 2C = 2.33 pg of DNA (Galbraith et al. 1983). However, taking into account the different values presented in the literature for chicken genome size (Greilhuber et al. 2007), we decided to calculate the size of the chicken blood used by our research group. For this purchase, *Pisum sativum* cv. 'Ctirad' (2C = 9.09 pg of DNA, Doležel et al. 1998; $n = 10$) and *Solanum lycopersicum* cv. 'Stupicke' (2C = 1.96 pg of DNA Doležel et al. 1992; $n = 10$) were used as internal standards. The new value for the CRBC used in our laboratories was 2C = 3.14 ± 0.155 pg with 2.93 and 3.43 pg as maximum and minimum values, respectively, (CV = 3.73%; $n = 20$). After that, plant and animal standards were compared with each other to calculate the nuclear DNA content in populations of *R. parnassifolius s.l.* and to test the reproducibility of FCM in the DNA genome size estimation. Table 4 shows the mean and standard deviation of the holoploid (2C) and monoploid (1Cx) nuclear DNA content in mass values (pg) of ploidy level with different

Table 4 Comparison of plant and animal DNA reference standards in cytotypes of *R. parnassifolius s.l.*

Cytotype	Standard	2C (pg)	Min.	Max.	Different standards	1Cx (pg)	CV (%)	*n*
Diploid	CRBC1	5.60 ± 0.227	5.25	6.33	a	2.80 ± 0.113	3.93	59
	PS	7.68 ± 0.372	6.99	7.97	b	3.84 ± 0.186	3.50	10
	SL	7.21 ± 0.391	6.77	8.01	b	3.60 ± 0.195	3.24	10
	CRBC2	7.55 ± 0.306	7.09	8.54	b	3.78 ± 0.153	3.93	59
Tetraploid	CRBC1	11.41 ± 0.382	10.93	12.48	a′	2.85 ± 0.095	4.03	49
	PS	14.99 ± 0.178	14.68	15.42	b′c′	3.74 ± 0.044	3.31	17
	SL	13.82 ± 0.305	13.08	14.46	c′	3.42 ± 0.200	3.40	22
	CRBC2	15.37 ± 0.514	14.75	16.84	b′	3.84 ± 0.129	4.03	49

Differences among standards (*Different standards*) within cytotypes were analyzed using a Kruskal–Wallis one-way ANOVA on ranks, and a Dunn's method. Standards identified by the *same letter* are not statistically different ($P < 0.001$)

CRBC1 Chicken red blood cells, 2C = 2.33 pg of DNA (Galbraith et al. 1983); *PS Pisum sativum* cv. 'Ctirad,' 2C = 9.09 pg of DNA (Doležel et al. 1998); *SL Solanum lycopersicum* cv. 'Stupicke,' 2C = 1.96 pg of DNA (Doležel et al. 1992); *CRBC2* chicken red blood cells, 2C = 3.14 pg of DNA (this study)

standards, and also the CV and minimum and maximum values obtained for each ploidy. Clear differences were observed when using different standards in the estimation of DNA content in absolute units, for example, comparing the Galbraith's value and the new value for the CRBC (Table 4). Although plant standards are preferable for determination of plant nuclear DNA content in absolute units (J Doležel, Institute of Experimental Botany, Olomouc, Czech Republic, personal communication), the new value for CRBC used in our laboratory is an adequate standard for ploidy determinations and is convenient because a single blood sample can be used for all runs.

Sample preservation

The feasibility and quality of FCM measurements largely depend on the species/tissue analyzed, the age of the material, and the storage conditions. Although fresh material is normally used, the potential use of frozen leaves and desiccated tissues may be an attractive alternative for many species and functions. It is also important to consider that, sometimes, the analysis of the tissue collection is quite difficult, especially when the samples are taken in distant areas, so the use of dehydrated and frozen plant tissues can be very useful for sample preservation in field botany. As a result, the relative nuclear DNA content of fresh plant material versus frozen and herbarium specimens from *R. parnassifolius s.l.* was analyzed (Table 5). As expected, peak attributes gradually changed with the type of material; fresh material provided smaller CVs and higher yields of intact nuclei while background fluorescence became more prominent with frozen and herbarium material. Analysis of fresh leaves typically yielded histogram results with a single prominent DNA peak, while histograms from desiccated tissues and frozen leaves presented peaks with poor resolution. The mean CV of fresh and non-fresh material ranged from 3.24 to 5.97% in diploid and from 3.31 to 5.24% in tetraploid. Fresh material showed less debris than that from the frozen/dehydrated samples, and the nuclei count was higher (Fig. 3a–d). The low nuclear count, the presence of debris, and the reported high CV for non-fresh material are presumably due to mechanical damage caused to the cells by storage at −80°C and possible interference by cytosolic compounds. However, the existence of debris did not affect the determination of DNA ploidy levels of the *R. parnassifolius s.l.* Herbarium vouchers of up to 6 months old seemed to be suitable for cytometric determination. However, in older individuals, more than 12 months old, or poorly preserved specimens, measurement results were highly degraded (high side scatter) or even lacked a DNA signal. Although desiccated tissues and frozen leaves of *R. parnassifolius s.l.* often experience a certain decrease in fluorescence intensity after several months of storage, the reliability of DNA ploidy value is not compromised and fluorescence intensity of isolated nuclei was highly comparable with that of fresh material, allowing reliable DNA ploidy estimation (Fig. 3a–d).

Morphometric measurements

Results of the exploratory data analysis of quantitative characters for diploid and tetraploid populations of *R. parnassifolius s.l.* from the Cantabrian Mountains are given in Table 6. The ranges of the measured characters of both DNA ploidy levels broadly overlapped, although showing significant differences between diploid and tetraploid cytotypes. These differences could be influenced by the date of sample collection. After using a one-way ANOVA procedure on all the morphological characters of each population (data not shown), the significant differences were attributable to ALB (Somiedo). These differences were detected between ALB and the rest of the

Table 5 Comparison of relative nuclear DNA contents from different sample preservation types of *R. parnassifolius s.l.*

Cytotype	Sample preservation	Standard	2C (pg)	Different sample	CV (%)	*n*
Diploid	Fresh material	SL	7.21 ± 0.391	a	3.24	10
	−80°C material (9 months old)	SL	7.25 ± 0.132	a	4.22	10
	Herbarium (6 months old)	SL	8.16 ± 0.519	b	5.97	5
	Herbarium (12 months old)	SL	No signal	–	–	5
Tetraploid	Fresh material	PS	14.99 ± 0.178	a′	3.31	17
	−80°C material (9 months old)	PS	16.44 ± 0.234	b′	4.93	10
	Herbarium (6 months old)	PS	16.27 ± 0.479	b′	5.24	5
	Herbarium (12 months old)	PS	No signal	–	–	5

Pisum sativum cv. 'Ctirad' was used as the standard tetraploid cytotype due to its genome size being close to that of *Ranunculus* samples. In the diploid cytotype, *Solanum lycopersicum* cv. 'Stupicke' was employed to avoid the peaks of the standard overlapping with the peaks of the unknown sample

Differences among sample preservation types (*Different sample*) within cytotypes were analyzed using a Holm–Sidak multiple comparison test. Standards identified by the *same letter* are not statistically different ($P < 0.001$)

PS P. sativum cv. 'Ctirad,' 2C = 9.09 pg of DNA (Doležel et al. 1998); *SL S. lycopersicum* cv. 'Stupicke,' 2C = 1.96 pg of DNA (Doležel et al. 1992)

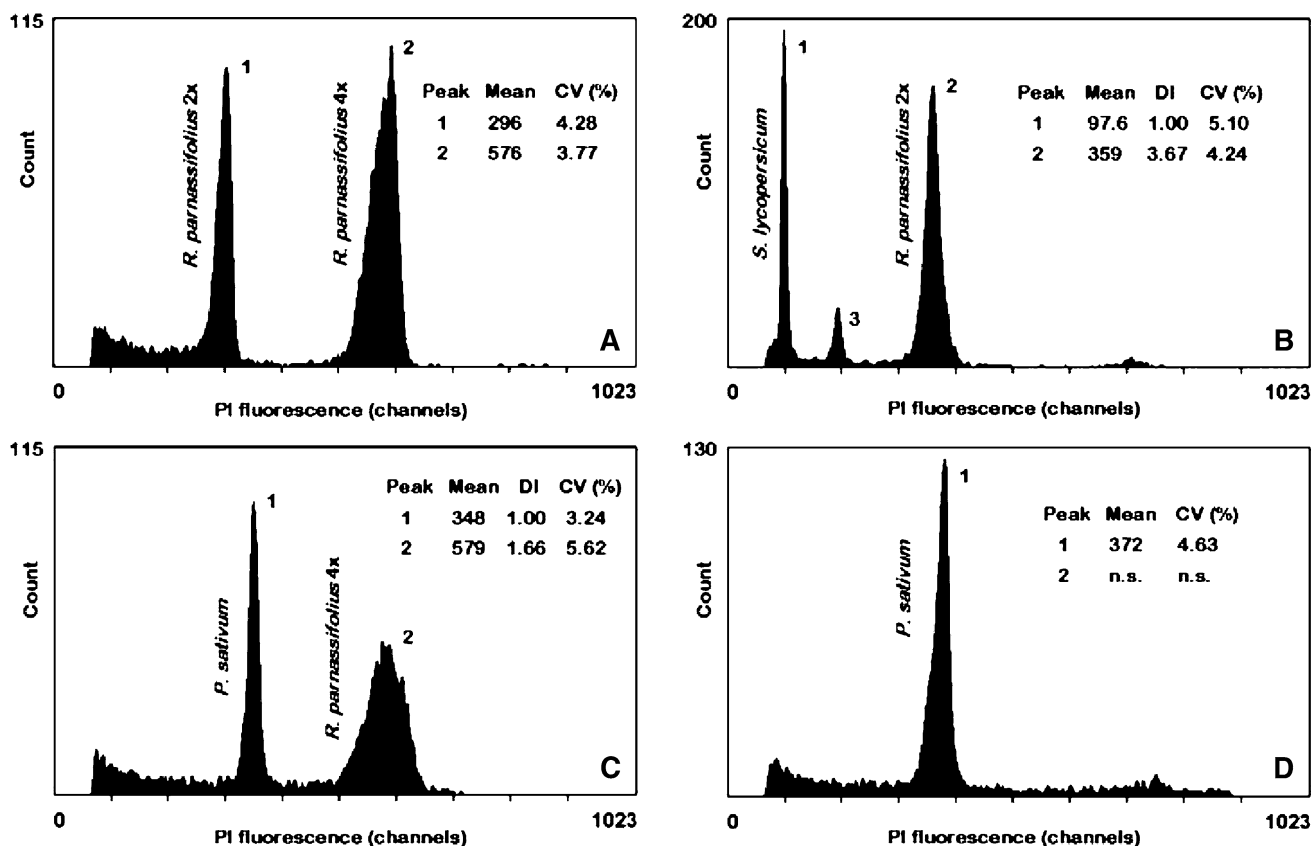

Fig. 3 Comparison of flow cytometric histograms obtained from relative nuclear DNA content of fresh, frozen, and herbarium-specimen materials from *R. parnassifolius s.l.* Changes in the quality of DNA signals and the reproducibility of the results are shown. Flow histograms show decreasing quality of signal and standard/sample ratio (**a** > **b** > **c** > **d**). **a** Simultaneous multi-ploidy analyses of fresh green leaves from CAN and CAR. **b** Frozen plant tissues (9 months old) from CAN. **c** Young herbarium specimens (6 months old) from GRA. **d** Damaged signal of old herbarium specimens (12 months old) from ALB. In the above histograms, the peaks are marked as follows: *1*, nuclei of internal standard at G0/G phase (except in histogram **a** where it corresponds to a multi-ploidy analysis); *2*, nuclei of sample at G0/G1 phase. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. In histogram **b**, the number *3* corresponds to G2/M peaks, and in histogram **d**, n.s. means "no signal"

Table 6 Results of exploratory data analysis of DNA ploidy level in *R. parnassifolius s.l.*

Character	Ploidy level	Mean	SD	Min.	5%	95%	Max.	*n*
LL	2*x*	1.57	0.29	1.10	1.12	2.06	2.47	86
	4*x*	1.75	0.58	0.80	0.90	2.88	3.43	66
LW*	2*x*	0.94	0.22	0.57	0.63	1.28	1.70	86
	4*x*	1.15	0.43	0.50	0.60	1.94	2.23	66
LS*	2*x*	0.48	0.09	0.31	0.36	0.71	0.71	40
	4*x*	0.56	0.13	0.36	0.37	0.76	0.77	17
WS*	2*x*	0.26	0.07	0.14	0.17	0.41	0.43	40
	4*x*	0.37	0.09	0.25	0.25	0.53	0.53	17
LP*	2*x*	0.78	0.11	0.55	0.64	1.00	1.10	39
	4*x*	1.12	0.29	0.56	0.57	1.58	1.60	13
WP*	2*x*	0.69	0.13	0.47	0.50	0.92	1.11	39
	4*x*	0.90	0.23	0.45	0.45	1.17	1.17	13
LA*	2*x*	0.24	0.04	0.15	0.17	0.30	0.32	49
	4*x*	0.27	0.04	0.20	0.20	0.30	0.30	21
WA*	2*x*	0.15	0.03	0.09	0.10	0.20	0.21	49
	4*x*	0.18	0.03	0.10	0.13	0.20	0.20	21
NF*	2*x*	2.97	1.70	1.00	1.00	6.00	7.00	76
	4*x*	2.17	1.40	1.00	1.00	5.00	7.00	46
NS	2*x*	5.00	0.00	5.00	5.00	5.00	5.00	40
	4*x*	5.00	0.00	5.00	5.00	5.00	5.00	17
NP*	2*x*	5.30	0.52	5.00	5.00	6.00	7.00	40
	4*x*	4.77	0.44	4.00	4.00	5.00	5.00	13
LLLW*	2*x*	1.73	0.35	0.90	1.18	2.37	2.59	86
	4*x*	1.57	0.28	1.06	1.15	2.03	2.25	66
LSWS*	2*x*	1.89	0.44	1.10	1.14	2.72	2.84	40
	4*x*	1.59	0.38	1.08	1.12	2.34	2.52	17
LPWP	2*x*	1.15	0.22	0.74	0.80	1.60	1.81	39
	4*x*	1.27	0.26	0.96	0.97	1.85	1.88	13
LAWA	2*x*	1.69	0.39	1.00	1.17	2.37	2.78	49
	4*x*	1.54	0.30	1.00	1.00	2.00	2.00	21

For character abbreviations, see Table 2

Differences between cytotypes were analyzed using a Mann–Whitney rank sum test. $*P < 0.001$

populations, and no differences were recorded within the Picos de Europa, regardless of their ploidy level. Using an ordination diagram of PCoA with qualitative and quantitative characters, based on Gower's similarity coefficient, diploids from the Picos de Europa and tetraploids from ALB samples showed distinct groupings (Fig. 4a). The first three axes accounted for 52.79% of the total variance (29.16, 13.27, and 10.35%, respectively). However, analyzing the ratio characters in the previously cited populations by PCA, the samples did not show distinct groupings and appeared to overlap (Fig. 4b) with 40.09% of the total variability explained by the first characteristic vector. In addition, the plots of the centroids of the *R. parnassifolius s.l.* samples, according to the two first dimensions of PCoA and including all vegetative and fructification characters, did not allow discrimination of the two cytotypes (Fig. 4c). The first three axes accounted for 64.06% of the total variance (32.31, 17.99, and 13.75%, respectively). Furthermore, similar values were obtained with PCoA based on all vegetative, fructification, and floral characters from populations of higher and lower altitudinal distribution (CAN and PAN), of which 75.65% of the variance was explained by three factors (37.82, 33.65, and 4.17%, respectively). Hence, both PCoA and PCA based on vegetative, floral, and fructification characters were performed on all the Cantabrian material analyzed, giving similar results. No morphological character or combination of characters could distinguish the plants as belonging to different ploidy levels. Similar results were obtained when analyzing the significantly different characters alone.

The *R. parnassifolius* group in the Cantabrian Mountains has similar morphotypes, with basal leaves that are subcordate-ovate and glabrous (except for the base and/or on the veins), glabrous or sparsely hairy sepals, smooth achenes, and in 96% of cases, the petals were white instead of pinkish white. Nevertheless some grouping tendencies can only be seen in the frequency of the occurrence of the number of stamens, e.g., tetraploid plants had a smaller number of stamens than diploid (see Fig. 2a, b). However, there were no events of aborted carpels, and the corolla was similarly regular in all the samples tested. We would also like to point out that the phenotypic uniformity in the field was consistent with the low variation reported between cytotype populations.

Discussion

Within the framework of broader taxonomic and evolutionary investigation of the *R. parnassifolius* group, we focused on unravelling the geographical distribution of ploidy levels both within and among populations in the Cantabrian Mountains, usually considered the western boundary of the European alpine chain. This area represents the southwestern boundary of the Atlantic biogeographical region (Ozenda and Borel 2000) and it has also been defined as a unique phytogeographical territory and is within the Eurosiberian region (Atlantic European province, Orocantabrian subprovince) (Rivas Martínez 2007). Prevailing climatic conditions, in both the past and present, have had a decisive effect on the flora and vegetation of the Cantabrian area (Fernández Prieto 1983). The existence of sympatric cytotypes of *R. parnassifolius s.l.* on the limestone territory of the Massif Central of the Picos de Europa (Küpfer 1974) raises some questions worthy of analysis. For the first time, nuclear DNA content for the *R. parnassifolius* group has

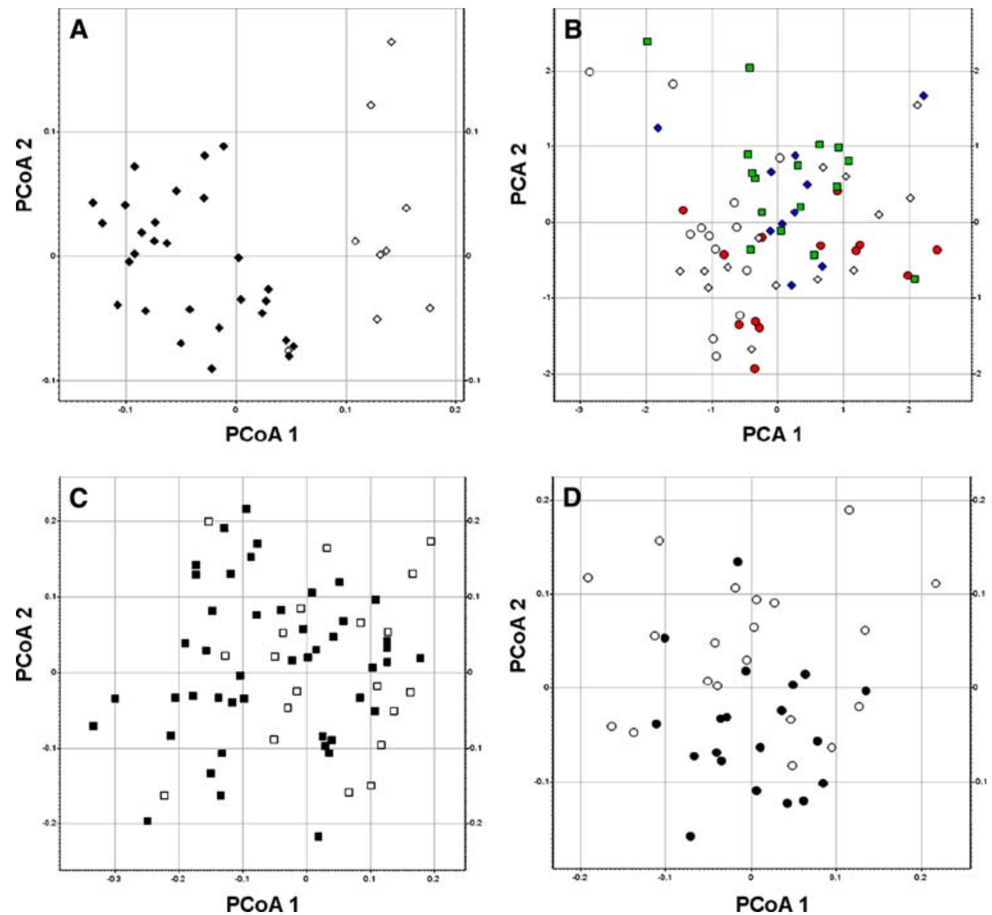

Fig. 4 Principal coordinate and components analyses of *R. parnassifolius s.l.* cytotypes based on morphological characters (quantitative, ratio, and binary). The diploid and tetraploid samples were found to be intermingled (except **a**). **a** Floral and fructification characters from diploids (Picos de Europa, represented as *filled diamonds*) and tetraploids (Somiedo, represented as *open diamonds*). **b** Ratio characters in the previously cited populations by PCA. The symbols correspond to BOC (*filled circles*), ALB (*open circles*), CAN (*filled diamonds*), PAN (*open diamonds*), and URR (*filled squares*). **c** Vegetative and fructification characters from diploid (*filled squares*) and tetraploid (*open squares*). **d** Vegetative characters between the populations of higher and lower altitudinal distribution sampled (CAN represented as *filled circles* and PAN represented as *open circles*)

been analyzed by using FCM. Our results are consistent with previous cytological data and with those provided by Goepfert (1974), who calculated the relative nuclear DNA content of one tetraploid sample of *R. parnassifolius* by cytophotometry with a value of $1C = 7.25$ pg (Bennett and Leitch 2004).

According to the basic chromosome number $x = 8$, it seems evident that the two *R. parnassifolius* groups are characterized by $2n = 16$ and $2n = 32$ chromosomes. Over the years there have been numerous studies that attempted to correlate genome size with various ecological parameters (e.g., latitude, altitude, elevation), but after comparing the results, their findings seem to be contradictory (Knight et al. 2005). An altitudinal distribution model has been proposed for this species by Küpfer (1974), as *R. parnassifolius* subsp. *heterocarpus* is distributed in an altitudinal range between 1,600 and 2,200 m, being replaced by the diploid *R. parnassifolius* subsp. *favargeri* at higher altitudes (i.e., 2,450 m, such as CAN). What it is more, Nava (1988), in the study of some orophyte taxa from the Picos de Europa, stated that the tetraploid taxa are the most frequent, the diploid cytotype being much rarer. However, and as mentioned by Laínz (1976), we have found there to be no clear altitudinal distribution model, with diploid samples being found in the less elevated populations studied (such as PAN) and the frequency of both cytotypes seems to be quite similar. On the other hand, we have in fact found a pattern of spatial distribution within the Picos de Europa with the diploid cytotype only being located in the Massif Central, while the tetraploid was present in the other massifs (Western and Eastern). It is worthy of note that this is the first time that mixed populations have been reported, demonstrating the co-existence of two different ploidies ($2x + 4x$ in the Picos de Europa-URR; $4x + 5x$ in Somiedo-ALB) in the Cantabrian Mountains. Until now, the existence of the pentaploid cytotype ($2n = 5x = 40$) had only been confirmed in two locations in the Central Pyrenees (Küpfer 1974; Vuille and Küpfer 1985). Previous studies by Küpfer (1974) revealed the existence of tetraploid plants (two counts) in Peña Vieja and its surroundings. In addition, Rivas Martínez et al. (1984) indicated the presence of *R. parnassifolius* subsp. *favargeri* (diploid) in the three massifs of the Picos de Europa. Based on this information, and considering the possible influence of plant phenology in the flowering of the two cytotypes, more comprehensive future samplings should be considered in such massifs, to determine a clearer distribution model.

The increased number of reports concerning mixed-ploidy populations highlights the interest generated in the evolutionary dynamics of polyploidy, as these populations provide conditions similar to the early stages of polyploid evolution (Suda et al. 2007a). The presence and extent of intraspecific variation in genome size has been a controversial topic over the last two decades (Doležel et al. 2007b), with intraspecific variation for some species having been verified (e.g., Creber et al. 1994; Sugiyama et al. 2002). However, the variability of nuclear DNA content within species has been dismissed by several reports applying better practice methodologies when conducting further investigations (Greilhuber 2005). Our data did not reveal a clear difference in monoploid genome sizes (1Cx) between diploid and tetraploid plants, supporting the findings of Smith and Bennett (1975), who suggested that the consequent doubling of the nuclear DNA content in tetraploid species resulted in minimal variation in Cx value.

For quite some time, there has been debate about the use of animal reference standards. In many studies, CRBC are used as the reference standard in FCM of nuclear DNA content in higher plants (Emshwiller 2002; Lim et al. 2006). However, their use has been called into question, mainly because there is no general agreement regarding the size and stability of the chicken genome and The Plant Genome Size Meeting in 1997 recommended not using it (http://data.kew.org/cvalues/homepage.html). Even at a suitable genome size ratio to the unknown sample, CRBC are not considered to be an ideal standard due to their strong DNA compaction (Hardie et al. 2002) and, more importantly, because of differences in their fixation and storage history compared to that of the sample under investigation. In addition, an instability in genome size has been reflected by the results of several studies, with 2C values ranging from 1.88 (Chen et al. 2002) to 3.01 pg (Johnston et al. 1999), with intraspecific variation and sex-related differences also being found. For instance, male and female chicken genomes differ by 2.7% because of sex chromosomes (Nakamura et al. 1990), which may introduce additional, though minor, experimental errors. However, animal reference standards, in the form of a nuclear suspension, are easy to use, they can be bought commercially or self-prepared, and can be fixed and stored, often for years, at low temperatures. Therefore, the use of CRBC as an internal standard is not totally reliable unless the genome size of chicken used in each individual study has been previously validated, and its use should be strongly discouraged in works where precise estimations of genome size in absolute units are required. Although the use of animal standards for plant samples may cast some doubt upon these estimates as absolute values, the use of a consistent methodology for all samples and the reproducibility of measurements of the same accessions, assuming the exact chicken genome size used is known, allow for comparisons of similarities and differences among species and populations. As such, we were able to obtain similar results using both animal and plant standards in the study of different populations of *R. parnassifolius s.l.*

Although it was originally developed for fresh plant tissues, Galbraith's protocol (Galbraith et al. 1983) would seem to be suitable for the estimation of relative nuclear DNA content in herbarium (Suda and Trávníček 2006a, b) or fixed specimens (e.g., Lucretti et al. 1999) and seeds (e.g., Sliwinska 2006). There has also been significant progress in the use of frozen plant tissues (e.g., Hopping 1993; Nsabimana and van Staden 2006), though some difficulties have been detected using these types of sample preservation. Firstly, analysis of samples from frozen and dehydrated tissues usually results in DNA content histograms of lower resolution and with excessive background from debris (Doležel et al. 2007a). Secondly, in measuring reliable nuclear DNA amounts, CVs of the peaks should not exceed 5.0% (Greilhuber et al. 2007), the problem being that these preservation techniques often result in samples that have resultant CVs over this limit. Other problems are due to the chemical composition of the cytosol, as plant cells produce a vast array of secondary metabolites that may interfere with a particular assay, for example with the staining of nuclear DNA. In addition, autofluorescence of some cellular components (chloroplasts and cell walls) may mask the weak fluorescence of stained targets (Loureiro et al. 2008).

The use of dry samples and frozen tissue in investigations would open up many new possibilities for plant research and significantly increase the power of FCM. The results of nuclear DNA content in herbarium samples of *R. parnassifolius s.l.* are consistent with those presented by Suda and Trávníček (2006b), where it was found that samples of *R. repens* analyzed with FCM gave no signal at 20 months old. While the preparation of herbarium specimens of *R. parnassifolius s.l.* up to 6 months of age showed a good signal, herbarium vouchers of more than 1 year exhibited unsatisfactory histogram quality. Furthermore, according to the same authors, the family Ranunculaceae is not FCM-friendly when estimating relative nuclear DNA content in dehydrated plant tissues (Suda and Trávníček 2006a). In recent years, the success of FCM studies utilizing desiccated plant material has increased considerably, but the use of frozen tissue has received less attention. In this study the feasibility of using *Ranunculus* leaves stored at −80°C for the analysis of ploidy using FCM with CV below 5.0% has been demonstrated. This fact is very important as the lifetime of deep freeze-stored samples is substantially longer than with other preservation methods, although the age limit of sample viability still needs to be

determined. That there is a slight variation in the genome size of frozen tetraploid material with respect to its fresh counterpart (which is not seen with diploid material) should also be mentioned. This could be due to a lower hardiness or tolerance to cold of tetraploid individuals compared with diploid plants as has been seen in other perennial species (e.g., Sugiyama 1998). The decrease in cold tolerance in tetraploids seems to result from changes in plant cells such as membrane fluidity, protein and nucleic acid conformation, and/or metabolite concentration (Chinnusamy et al. 2007). Moreover, the aggregation of water molecules to form a stable ice nucleus, sometimes associated with coatings of debris, could interfere with staining and/or the fluorescence of the fluorochromes. The aggregation of minor particles with nuclei can play a key role in this interference and can even lead to an apparent increase in nuclear fluorescence (Loureiro et al. 2006a). Although a small decrease in DNA signal in herbarium specimens (under 6 months old) and frozen material compared to fresh material is documented, both stored specimen types have proved satisfactory in the determination of ploidy level. The use of both sample preservation methods opens up new perspectives for analyzing field-collected material, and this advance greatly expands the potential application of FCM in botany, where samples frequently cannot be immediately analyzed.

The co-occurrence, frequency distribution, and origins of infraspecific cytotypes are challenging topics that have not been thoroughly explored. Recent studies have indicated that co-distribution of cytotypes may be more widespread than had been previously expected (e.g., Rosenbaumová et al. 2004; Suda et al. 2007b; Španiel et al. 2008; Kolář et al. 2009). Although only very limited information concerning potential sympatric growth of plants with different ploidy levels and their distribution pattern at fine spatial scales is available, the current work depicts the landscape patterns of the calcareous screes in the Cantabrian Mountains. Our recordings of *R. parnassifolius* subsp. *favargeri* and *R. parnassifolius* subsp. *heterocarpus* from apparently identical habitats within a geographically limited area, like the Picos de Europa, indicate that their niches could overlap. Whilst DNA ploidy differences were found, it is important to acknowledge that at the morphological level, the data were not reliable enough to enable the differentiation of the two cytotypes of *R. parnassifolius* in the Cantabrian Mountains. Some recent studies have identified similar cases, where, despite slight morphological tendencies expressed at the population level, the cytotypes cannot be reliably distinguished morphologically, thus concluding that the coexistence of different cytotypes within a single species, without recognition of infraspecific taxa, is rather frequent (e.g., Perný et al. 2008; Španiel et al. 2008; Zonneveld 2008).

This work represents the first study dealing with FCM in *R. parnassifolius* and has found the use of FCM in ploidy estimation to be reliable and efficient, suggesting it may prove to be a significant tool for the delimitation of those cytotypes that are sometimes not readily separated by morphoanatomic studies. In light of the results obtained in this work, new issues are raised, such as the distribution of both taxa in other regions such as the Pyrenees and the Alps, or whether the distribution of polyploid phenomena is associated with apomixis as in other species within the same genus (Paun et al. 2006). Moreover, genome size data are helpful in generating or supporting hypotheses about parental taxa and, consequently, about allo- versus auto-polyploid origins. We conclude that the existence of mixed-cytotype populations along the study area is not common and the frequency of diploid and tetraploid cytotypes seems to be quite similar. Furthermore, we have not found evidence supporting a model of altitudinal distribution between the two cytotypes. These data are therefore important for establishing strategic priorities for conservation programs throughout the Cantabrian Mountains in general, and more specifically, for intensifying the strategies implemented in the Picos de Europa.

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