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Notes on genome size in the hybrid *Ranunculus* × *luizetii* (Ranunculaceae) and its parents by flow cytometry

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Abstract
Notes on genome size in the hybrid *Ranunculus* × *luizetii* (Ranunculaceae) and its parents by flow cytometry.- Flow cytometry was used to estimate the nuclear DNA content in the natural hybrid *Ranunculus* × *luizetii* and its parents. Our results indicate that the genome size of the hybrid *R.* × *luizetii* is closer to *R. pyrenaeus* than to *R. parnassiifolius*, providing an evidence of genome downsizing.

Key words: C-value; flow cytometry; genome size; nuclear DNA amounts; *Ranunculus*.

Resumen
Notas sobre el tamaño del genoma en el híbrido *Ranunculus* × *luizetii* (Ranunculaceae) y sus progenitores mediante citometría de flujo.- Se ha empleado la citometría de flujo para estimar el contenido de ADN nuclear en el híbrido *Ranunculus* × *luizetii* y sus progenitores. Nuestros resultados indican que el tamaño del genoma del híbrido *R.* × *luizetii* se acerca más a *R. pyrenaeus* que a *R. parnassiifolius*, con una evidencia de reducción del genoma.

Palabras clave: cantidades de ADN nuclear; citometría de flujo; *Ranunculus*; tamaño del genoma; valor C.

Since the first report on flow cytometry (FCM) analysis of plant material 38 years ago (Heller, 1973), applications of FCM in plant population and evolutionary biology have expanded dramatically. Nuclear DNA content, in combination with other morphological and molecular characters, can contribute to intergeneric classification, taxa delimitation or hybrid identification (Doležel *et al.*, 2007). In the present work, hybrid detection using FCM has been employed to study the natural hybrid *Ranunculus* × *luizetii* Rouy (*R. parnassiifolius* subsp. *parnassiifolius* L. × *R. pyrenaeus* L.) collected from the Pyrenees [Spain, Lérida, Espot, Coll de la Creu de l'Eixol, 43º 10' 35.4'' N / 4º 49' 24.1'' W, 2207 m, E. Cires & J. A. Fernández Prieto, 31975 FCO] in the same area where it was described by Rouy (1893). This study might contribute to a better understanding of zones of overlap between rare and closely related species, with potential applications to the field of conservation biology. Samples for FCM measurements were prepared from fresh tissues of young leaves of *R.* × *luizetii* and its parental taxa *R. pyrenaeus*; regarding *to R. parnassiifolius*, the FCM data come from Cires *et al.* (2010). Nuclear DNA amounts were estimated

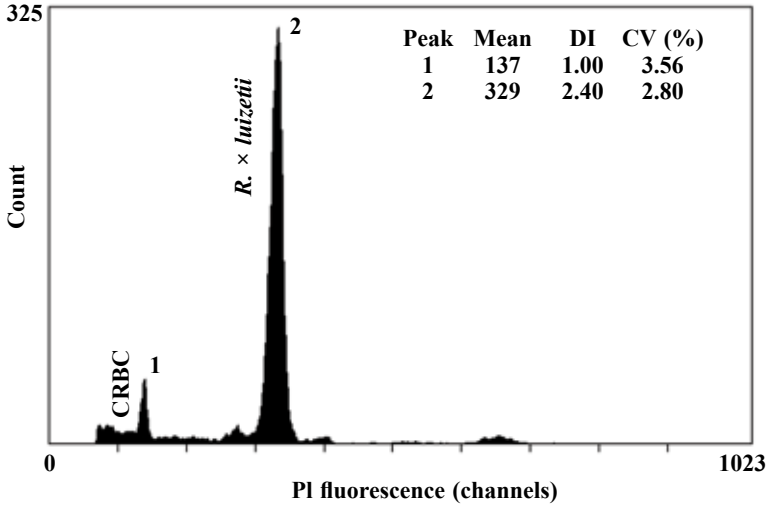

Figure 1. One of the flow cytometric histograms obtained for *Ranunculus* × *luizetii*. The peaks marked with 1 and 2 indicate nuclei at the G0/G1 phase of the internal standard and the G0/G1 phase of the sample, respectively. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. Internal reference standard: CRBC: chicken red blood cells, 2C = 3.14 pg of DNA (Cires *et al.*, 2009).

with propidium iodide staining, 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, Inc. 2006), following the protocol described by Cires *et al.* (2009, 2010). As a reference, unstained chicken red blood cell (CRBC) nuclei were added to the isolation buffer to ensure the ploidy level of parental plants. The 2C DNA content (2C = holoploid genome size; Greilhuber *et al.*, 2005) was calculated as:

$$\frac{\textit{Ranunculus}\text{ sp. G0/G1 peak mean}}{\text{Reference standard G0/G1 peak mean}} \times \text{nuclear DNA content of reference standard}$$

The monoploid genome size (1Cx; *sensu* Greilhuber *et al.*, 2005) of all plants was also calculated in mass values (pg) and Mbp, and at least 5000 nuclei were analysed per sample.

FCM analyses of *Ranunculus* × *luizetii* (Fig. 1) and its parental species resulted in high resolution histograms with mean coefficient of variation of G0/G1 peaks (interphase nuclei of cycling cells), ranging from 2.50 to 3.60%. Nuclear DNA contents are shown in Table 1. The 2C nuclear DNA content of 27 specimens of *R.* × *luizetii* and its parental taxa were determined using FCM, providing histograms with well-defined peaks of both sample and internal reference standards (CRBC) (Table 1). Statistical analyses revealed significant differences ($P < 0.05$) between the 2C DNA content of *R.* × *luizetii* and one of its parents, *R. parnassiifolius*. However, significant differences were not found if we take into account the other parental species, *R. pyrenaeus* (see Table 1).

In recent years, molecular approaches have suggested several mechanisms (*e.g.* elimination of non-coding and coding DNA sequences, homologous and illegitimate recombination, etc.) to explain genome downsizing in hybrids and/or polyploids (*e.g. Brassica* (Song *et al.*, 1995); *Nicotiana* (Petit *et al.*, 2007); *Aegilops* and *Triticum* (Eilam *et al.*, 2008), as these mechanisms have been shown to operate in both diploids and polyploids (Leitch & Bennett, 2004). The interspecific hybridization event in *Ranunculus* × *luizetii* has been supported by morphological and molecular data (see Rouy, 1893; Cires *et al.*, unpublished data). Indeed, Amplified Fragment Length Polymorphism (AFLP) and Internal Transcribed Spacers (ITS) of ribosomal DNA revealed genetic intermediateness between the hybrid samples (*R.* × *luizetii*) and the parental taxa; additionally a decrease in pollen fertility

Table 1. Nuclear DNA contents of *Ranunculus* × *luizetii* and its parental species using FCM. The values are given as means with standard deviation of the mean (SD) of the nuclear DNA content (pg/2C). The 2C range of values (min.- minimum, max.- maximum) obtained for each population, the monoploid nuclear DNA content (1Cx) in mass values (pg) and Mbp, the mean coefficient of variation (CV, %), and the number of individuals measured (n) are also given.

		Nuclear DNA Content						
			2C range					
Taxon	**Ploidy Level**	**2C (pg)**	**Min.**	**Max**	**1Cx (pg)**	**1Cx (Mbp)***	**CV(%)**	**n**
R. parnassiifolius †	2x	8.47 ± 0.528 a	7.50	8.90	4.23 ± 0.264 a	4,144 ± 258.192	3.60	13
R. × luizetii	2x	7.40 ± 0.290 b	7.10	7.82	3.70 ± 0.145 b	3,622 ± 141.567	3.10	7
R. pyrenaeus	2x	7.55 ± 0.083 b	7.43	7.66	3.77 ± 0.041 b	3,693 ± 40.694	2.50	7

Differences between populations were analyzed using a one-way ANOVA procedure and Dunn's method for pair-wise comparison. Means followed by the same letter are not statistically different ($P < 0.05$).

Internal reference standard: CRBC 2C = 3.14 pg of DNA (Cires *et al.*, 2009).

† This information was previously published in Cires *et al.* (2010).
* 1 pg = 978 Mbp (Doležel *et al.*, 2003).

from the hybrid was observed (Cires *et al.*, unpublished data). Although an intermediate genome size between the parents should be expected, FCM results indicate that the genome size of the hybrid *R. × luizetii* is closer to that of *R. pyrenaeus* than to that of *R. parnassiifolius* with an evidence of genome downsizing, and suggest that loss of DNA amount following hybrid formation may be a phenomenon more common than expected.

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