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Evaluation of polysomaty and estimation of genome size in *Polygala vayredae* and *P. calcarea* using flow cytometry

Sílvia Castro [a,b,*], João Loureiro [b], Eleazar Rodriguez [b], Paulo Silveira [b], Luis Navarro [a], Conceição Santos [b]

[a] *Department of Plant Biology and Soil Sciences, Faculty of Science, University of Vigo, 36200 Vigo, Spain*
[b] *CESAM and Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal*

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Abstract

In seed plants, endopolyploidy is regarded as a common and developmentally regulated phenomenon. However, in Polygalaceae polysomaty has never been studied. In this work the endopolyploidy of *Polygala vayredae* ($2n = 28$, subgenus *Chamaebuxus*) and *P. calcarea* ($2n = 34$, subgenus *Polygala*) was evaluated using flow cytometry. With this technique it was possible to observe polysomaty in endosperm, leaves and petals of both species, although with different patterns. Usually, in *P. vayredae*, 2C and 4C ploidy levels were detected while for leaves of *P. calcarea*, an extra 8C level was observed. In *P. vayredae*, statistically significant differences were observed in the endopolyploid level between fully expanded young leaves and 1-year-old mature leaves. Nuclear DNA content analysis in these taxa revealed significant differences, with *P. vayredae* presenting a higher genome size (2C = 2.71 pg DNA) than *P. calcarea* (2C = 0.98 pg DNA). These data and the highest level of polysomaty found in *P. calcarea* seem to point to a negative correlation between genome size and endopolyploidy, as observed in other works.

Keywords: Endopolyploidy; Flow cytometry; Genome size; *Polygala* spp.; Polysomaty

1. Introduction

Polygala L. (Polygalaceae) comprises about 725 species largely distributed all over the world that present a high diversity of life forms and adaptive strategies, occupying a wide range of ecological niches [1]. The species studied in this work, *P. vayredae* Costa (subgenus *Chamaebuxus* (DC.) Schb.) and *P. calcarea* F. W. Schultz (subgenus *Polygala* Duch.), are either small perennial herbs or shrubs, with alternate entire leaves and production of renewal stems during flowering. However, these species present very distinct ranges of distribution. While *P. vayredae* is a narrow endemic species from oriental pre-Pyrenees (with a distribution area of approximately 13 km^2) with high conservation interest [2], *P. calcarea* is widespread throughout Western Europe, northwards to South England [3].

Endoreduplication, which consists of repeated cycles of DNA synthesis without occurrence of cell divisions, is a common phenomenon in differentiated cells of seed plants [4,5]. This process leads to the presence of cells with various ploidy levels in an organ, i.e., polysomaty [5]. The patterns of polysomaty, within a species, are usually different in various organs and correlated with the developmental stage [6–9]. Despite being a common and frequently studied phenomenon, the biological significance of endopolyploidy is not yet understood [5,10]. Some correlations between systematics, organ, life strategy, genome size, cell size and nuclear volume were already found [11–13]. Barow and Meister [11] suggested that phylogenetic position is the major factor determining the degree of endopolyploidy within a species, while organ type, life cycle and nuclear DNA content have a minor but also important effect on endopolyploidization. So far, systemic endopolyploidy was mainly described for species with small genomes including *Arabidopsis thaliana* [14], *Beta vulgaris* [5], *Brassica oleracea* [12,15–17], *Cucumis sativus* [8], *Zea*

* Corresponding author at: Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.
Tel.: +351 234 370 350; fax: +351 234 426 408.
E-mail address: scastro@bio.ua.pt (S. Castro).

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mays [18], *Lycopersicon esculentum* [4], *Medicago sativa* and *M. truncatula* [19], and several species of Orchidaceae [20–23] and succulents [7,10,24]. Recent work by Barow and Meister [11], revealed the occurrence of polysomaty in several organs of 33 out of 54 seed plant species, belonging to 10 angiosperm families.

In the interesting Polygalaceae family, polysomaty has never been studied. Nevertheless, in the evolutionarily related family Fabaceae [25] various degrees of endopolyploidy in different organs were described for *M. sativa* and *M. truncatula* [19] and for *Glycine max*, *Phaseolus vulgaris*, *Pisum sativum*, *Vicia faba* and *Trifolium repens* [11]. Also, only recently the genome size of a species belonging to this family (*P. calcarea*) has been analysed [26].

Flow cytometry (FCM) has been used to analyse endopolyploidy in plants since the beginning of 1990s [7]. The advantages of this technique, i.e., a quick and accurate estimation of the relative nuclear DNA content of large numbers of nuclei within a tissue/organ [27,28], surely increased the interest to study this phenomenon. Previous methods, as Feulgen densitometry, are time consuming, limited to a single tissue for a given plant and quantify relatively low numbers of nuclei [15].

Due to the still reduced information about this interesting phenomenon, more studies reporting the presence/absence of endopolyploidy in seed plants are needed. Therefore, the aim of this work was to: (1) evaluate polysomaty in various organs of *P. vayredae* and *P. calcarea,* two congeneric species differing in their distribution range; (2) increase knowledge on the degree of endopolyploidy in the explant tissue source, in view with the conservation interest of *P. vayredae*, and (3) contribute to increase the information on endopolyploidy in seed plants. The 2C nuclear DNA content of these species was also estimated using flow cytometry.

2. Materials and methods

2.1. Plant material

Fresh plant material and seeds were directly collected in Alta Garrotxa region (Catalunya, Spain) from field growing plants. Four populations of *P. calcarea* were sampled; two were located in Colldecarrera and the other two in Serrat dels Boixos. *P. vayredae* was collected in two populations of *P. calcarea* (one from each location). All the populations correspond to mesophytic meadows (*Mesobromion*) with *Pinus sylvestris* and *Buxus sempervirens*. Fresh plant material was maintained in moistened paper, enclosed in plastic bags and analysed within 2–3 days. Cotyledons, endosperm, fully expanded young leaves (produced in the current year), mature leaves (1-year-old) and petals were evaluated for polysomaty.

2.2. Polysomaty evaluation using flow cytometry

Cotyledons and endosperm were dissected from seeds using fine needles over a binocular microscope. The total amount of material per seed (approximately 3–10 mg) was used for FCM analysis. For leaves and petals, approximately 100 mg of plant material was weighed. Plant material was prepared according to the protocol of Galbraith et al. [29]. Briefly, the tissue was chopped in a glass Petri dish with a razor blade in about 1 ml cold Tris·$MgCl_2$ buffer (200 mM Tris, 4 mM $MgCl_2 \cdot 6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5 [30]) modified with 1% (w/v) polyvinylpyrrolidone 10 (PVP-10). Nuclear suspensions were filtered through an 80 μm nylon filter into an ice-cold sample tube and supplemented with 50 μg/ml of propidium iodide (PI, Fluka, Buchs, Switzerland) for DNA staining. RNAse (Fluka, Buchs, Switzerland) at 50 μg/ml was added to prevent staining of double stranded RNA.

Samples were incubated on ice for a 10–15 min period, after which they were analysed in a Coulter EPICS XL (Beckman Coulter, Hialeah, FL, USA) flow cytometer equipped with an air-cooled argon-ion laser (JDS Uniphase, San José, CA, USA) operating at 488 nm. PI fluorescence emitted from nuclei was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter and converted to 1024 channels. Before starting the analysis, the instrument was checked for linearity with fluorescent check beads (Beckman Coulter, Hialeah, FL, USA). Fluorescence histograms were plotted in both linear and logarithmic scale and several regions were defined in PI pulse integral *versus* PI pulse height and side scatter (SS) *versus* PI pulse integral cytograms, to eliminate doublets (see Fig. 1 for an example) and reduce counts of background debris, respectively. At least 5000 nuclei were analysed per sample. With the exception of *P. calcarea* petals, where only one measurable sample was obtained, 9–11 replicates were performed per organ.

For each sample the percentage of nuclei present in each peak was calculated using the SYSTEM II v.3.0 software (Beckman Coulter, Hialeah, FL, USA). Also, and as a measure of endopolyploidization, the cycle value of each sample was estimated using the following formula:

$$\text{cycle value} = \frac{0 \times n_{2C} + 1 \times n_{4C} + 2 \times n_{8C} + 3 \times n_{16C} \ldots}{n_{2C} + n_{4C} + n_{8C} + n_{16C} \ldots} \quad (1)$$

with n_{2C}, n_{4C}, n_{8C},. . ., (n_{xC}), being the number of nuclei with C-level 2C, 4C, 8C,. . ., *X*C.

This value was first defined by Barow and Meister [11] and indicates the mean number of endoreduplication cycles per nucleus. Organs with cycle values above 0.1 are considered endopolyploid.

2.3. Genome size estimations using flow cytometry

For nuclear DNA content estimations of *P. vayredae* and *P. calcarea*, leaves from both sample and internal reference standard (*L. esculentum* cv. Stupicke having a 2C nuclear DNA content of 1.96 pg as determined by Doležel et al. [31] for *P. vayredae*, and *Z. mays* cv. CE-777 with a 2C nuclear DNA content of 5.43 pg according to Lysák and Doležel [32]

for *P. calcarea*) were analysed using the protocol described above.

Analyses of five individuals per population of *P. vayredae* and three to four per population of *P. calcarea* were performed within 2 days to minimize the variation related with the instrument. Two to three replicates were performed per individual.

Absolute nuclear DNA estimations of *Polygala* spp. in pg were performed using the formula:

$$\text{Polygala sp. 2C nuclear DNA content (pg)} = \frac{\text{Polygala sp. } G_0/G_1 \text{ peak mean}}{\text{reference standard } G_0/G_1 \text{ peak mean}} \times 2\text{C nuclear DNA content of reference standard} \quad (2)$$

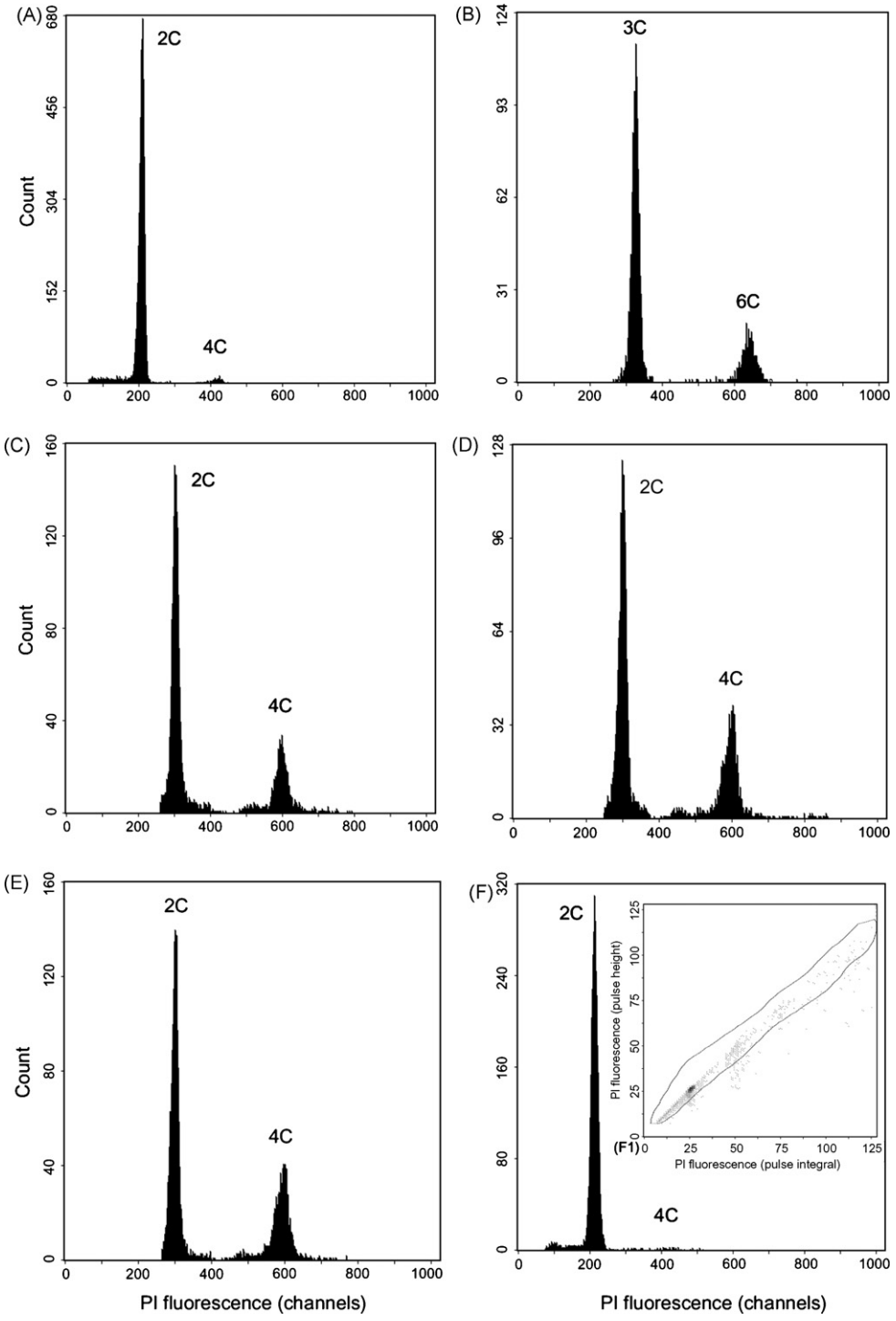

Fig. 1. Flow cytometric evaluation of polysomaty in *P. vayredae* (A)–(E) and *P. calcarea* (F)–(J). Histograms of relative fluorescence intensity of nuclei isolated from: cotyledons ((A) and (F)); endosperm ((B) and (G)); fully expanded young leaves ((C) and (H)); 1-year-old mature leaves ((D) and (I)) and petals ((E) and (J)). In histogram (F), the inset represents a PI pulse integral *versus* PI pulse height cytogram where a gating region was defined to exclude doublets and background debris. A similar gating was applied to all histograms of relative fluorescence intensity.

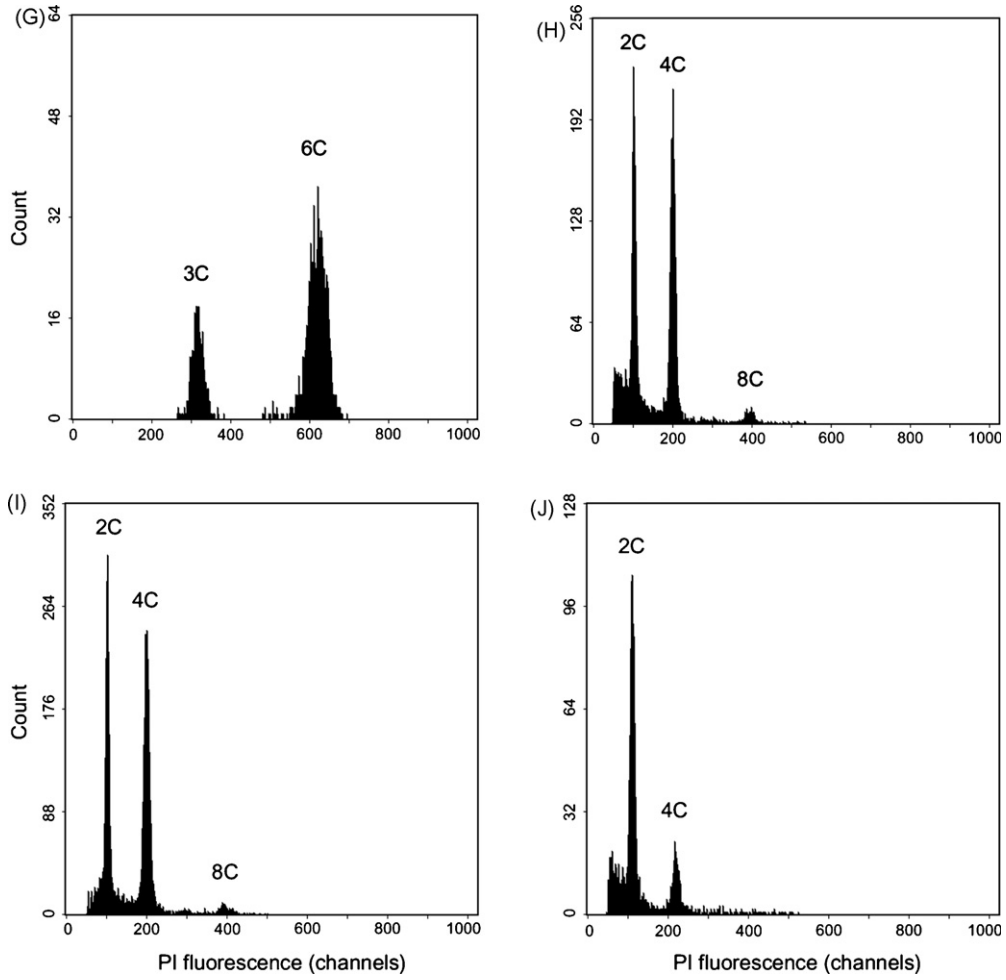

Fig. 1. (*Continued*).

Mass values (pg) were converted into number of base-pairs (bp) using the factor: 1 pg = 0.978 × 10^9 bp [33].

2.4. Statistical analyses

In both species, differences in cycle value data among organs were analysed using a one-way ANOVA (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA) procedure. A Tukey–Kramer multiple comparison test was used for pair-wise comparison.

The same test was applied for each species to analyse differences in genome size among populations.

3. Results and discussion

Endopolyploidy is of widespread occurrence in seed plants, but apparently restricted to certain phylogenetic groups [6,11]. Flow cytometric analysis of *P. vayredae* and *P. calcarea* plants showed the occurrence of polysomaty in several organs (Table 1 and Fig. 1). This is the first report of this phenomenon in Polygalaceae.

Different patterns of polysomaty were observed in the studied species. For both, polysomaty has been detected in all of the analysed organs with exception of the cotyledons (Table 1 and Fig. 1). However, cycle values obtained in *P. vayredae* were usually considerably lower. In this species, only 2C and 4C nuclei (leaves and petals) or 3C and 6C nuclei (endosperm) were obtained, with most of the nuclei presenting 2C (61.9–70.7%, Fig. 1C–E) or 3C ploidy level (81.4%, Fig. 1B), respectively. Additionally, the developmental stage of the leaves was an important factor, with statistical significant differences ($P < 0.05$) obtained between fully expanded young leaves and 1-year-old mature leaves with the latter having a higher percentage of 4C nuclei (38.0%) in comparison with young leaves (25.4%). Petals presented a similar cycle value as fully expanded young leaves (no statistical significant differences were observed, $P < 0.05$), while the endosperm presented the lowest polysomaty level. Differences in the levels of polysomaty between organs with different development stages were already reported in other species, with older tissues often possessing nuclei of higher ploidy levels within the same plant [7,10,15,22,23]. In *P. calcarea*, significantly higher polysomaty levels were obtained in leaves and endosperm (cycle value >0.617, Table 1) with most of the cells presenting 4C (53.5–53.7%, Fig. 1H and I) or 6C ploidy level (61.7%, Fig. 1G), respectively. Additionally, in leaves an extra 8C ploidy level was detected (Table 1 and Fig. 1H and I). Contrarily to what was observed in *P. vayredae*, the DNA

Table 1
DNA ploidy patterns in percentage of nuclei for different organs of *Polygala vayredae* and *P. calcarea*

Species	Organ	Ploidy patterns (% percentage of nuclei population)					Cycle value[a,b]
		2C	3C	4C	6C	8C	
Polygala vayredae	Cotyledons	96.6 ± 1.68	–	3.4 ± 1.73	–	–	0.034 ± 0.0168 a
	Endosperm	–	81.4 ± 5.88	–	18.6 ± 5.92	–	0.186 ± 0.0588 b
	Fully expanded young leaves	74.6 ± 3.84	–	25.4 ± 3.91	–	–	0.266 ± 0.0383 c
	1-year old mature leaves	61.9 ± 8.07	–	38.1 ± 8.12	–	–	0.383 ± 0.0802 d
	Petals	70.7 ± 1.76	–	29.3 ± 1.78	–	–	0.293 ± 0.0174 c
Polygala calcarea	Cotyledons	90.7 ± 3.55	–	9.3 ± 3.60	–	–	0.093 ± 0.0355 a
	Endosperm	–	38.3 ± 8.56	–	61.7 ± 8.53	–	0.617 ± 0.0856 b
	Fully expanded young leaves	41.2 ± 9.00	–	53.5 ± 8.04	–	5.3 ± 1.92	0.642 ± 0.1024 b
	1-year old mature leaves	41.3 ± 11.69	–	53.7 ± 10.70	–	5.0 ± 3.32	0.638 ± 0.1346 b
	Petals	78.6	–	21.4	–	–	0.214

Values are given as mean and standard deviation of the mean of ploidy patterns and cycle value.
[a] Cell cycle value was calculated according to Barow and Meister [11]. Organs with cycle values above 0.1 are considered endopolyploid.
[b] For each species, values followed by the same letters (a or b) are not significantly different according to the multiple comparison Tukey–Kramer test at $P < 0.05$.

ploidy patterns in leaves of different age were highly similar (no statistical significant differences were obtained at $P < 0.05$). Petals of *P. calcarea* presented a lower cycle value with most of the cells in 2C and with no cells in 8C (Fig. 1J). The occurrence of endopolyploidy in floral organs has been frequently detected in several plant species (e.g. [9,11,17]). Nevertheless, in other species (e.g., *Vanda hookeriana* × *Vanda teres* [22]) endoreduplication was not observed in flowers. With respect to seeds, it seems that endopolyploidy is prevalent in large seeds of endopolyploid species (e.g., *Z. mays*, *P. sativum*, *V. faba*, *Cucurbita* sp. [11]), rather than in small ones (e.g., *A. thaliana* [34]). However, in both cases, endopolyploidy tends to increase in cotyledons during germination [8,11].

In comparison with the endopolyploidization studies already performed in Fabaceae, the closest phylogenetic family, the patterns of polysomaty obtained by Barow and Meister [11] for *G. max*, *P. vulgaris*, *P. sativum* and *V. faba*, were different from those obtained in our work. In these species the higher cycle values were obtained for cotyledons and petals, while leaves cycle values were among the lowest. As Barow and Meister [11] investigated cotyledons after germination, the different degrees of endopolyploidy before and after germination in some species could be one possible reason for the relatively low endopolyploidization in *Polygala*. In spite of the fact that the phylogenetic position, namely at family level, has a major impact on the occurrence of polysomaty, different species belonging to the same family may exhibit different patterns of polysomaty. This seems to be the case for the two species studied here, where genome size appears to have a higher impact on endopolyploidy than phylogenetic position.

No significant differences were found in the polysomaty level of individuals among the studied populations of each species. Previous works have shown that some environmental conditions (e.g., light, nutrient supply, temperature, parasites) may have an influence on the degree of endopolyploidy (for a review see Barow [6]). From our data and considering that the analysed individuals of *P. vayredae* were growing in *P. calcarea* populations, it seems that the abiotic and biotic conditions of each population were not the reason for the different extent of polysomaty that was observed.

Whilst searching for the biological significance of polysomaty, recent works pointed out the importance of a positive correlation between DNA content and volume of endopolyploid nuclei and cells [6,11]. In these investigations it has been suggested that this phenomenon may be important in the acceleration of growth of endopolyploid plants and in the physiological function of a given cell. It was also suggested that the occurrence of endopolyploidy in endosperm (as found here for *P. calcarea*), that implies the presence of cells with a larger volume, has the advantage of saving material and time during tissue development [6].

The quality of the FCM histograms (in linear scale), as a result of the coefficient of variation (CV) of 2C and 4C peaks and background debris, depended more on the species than on the organ analysed. Histograms obtained from *P. vayredae* tissues presented CV values ranging from 1.89% to 3.90% (mean CV = 2.99%) for the 2C peak and from 1.46% to 3.63% (mean CV = 1.90%) for the 4C peak. In the case of *P. calcarea*, CV values were slightly higher, ranging from 2.93% to 5.04% (mean CV = 4.35%) for the 2C peak and from 2.69% to 4.01% (mean CV = 3.16%) for the 4C peak. In any sample, 4C peaks had lower CV values in comparison with 2C peaks. This is due to channel position of each peak, as in an analogue-to-digital conversion system (like the one present in our flow cytometer), the resolution increases with the magnitude of the signal, thus the lower the channel, the higher the CV value [35]. The analysis of this parameter for each organ revealed that in these species, the CV values were usually not significantly different among organs ($P < 0.05$). As it can be seen in Fig. 1, the gating of several regions was a useful approach to eliminate doublets and background debris from analysis. In this way, the effect of these particles in relation to the total number of nuclei in each peak was minimized.

Genome size estimations of *P. vayredae* and *P. calcarea* are given in Table 2 and illustrated in Fig. 2. Within each species no statistical significant differences were detected among populations ($P < 0.05$), revealing high homogeneity among

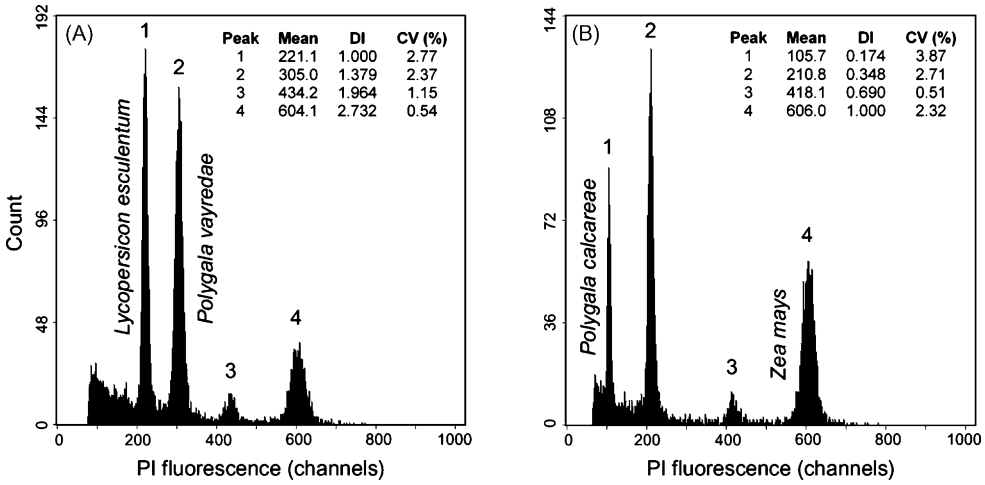

Fig. 2. Flow cytometric estimation of genome size of *Polygala vayredae* (A) and *P. calcarea* (B). Histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Polygala* spp. and the internal reference standard (*L. esculentum* cv. Stupicke, 2C = 1.96 pg DNA, for *P. vayredae*; *Zea mays* cv. CE-777, 2C = 5.43 pg, for *P. calcarea*). In histogram A, peaks 1 and 3 correspond to 2C and 4C nuclei of *L. esculentum* and peaks 2 and 4 correspond to 2C and 4C nuclei of *P. vayredae*. In histogram B, peaks 1, 2 and 3 correspond to 2C, 4C and 8C nuclei of *P. calcarea*, while peak 4 refers to 2C nuclei of *Z. mays*. The mean channel number, DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation (CV, %) value of each peak is also given.

individuals in the studied region. At species level, genome size estimations revealed that despite belonging to the same genus, the studied species present distinct genome sizes, with *P. calcarea* having a smaller genome (almost 1/3) than *P. vayredae*. The analysis of the genome size in the light of the data of polysomaty appears to be in accordance with the previously established negative correlation between both variables [7,11].

The smaller genome size of *P. calcarea* despite its larger chromosomes number (*P. calcarea*: 34 chromosomes [3], *P. vayredae*: 28 chromosomes [36]) results from considerably smaller chromosomes in *P. calcarea* than in *P. vayredae*. Indeed, these species belong to two different subgenera within *Polygala*: *P. vayredae* belongs to *Chamaebuxus* (DC.) Schb. and *P. calcarea* to *Polygala* Duch. Besides morphological characters, palynological analysis in *Polygala* spp. from Iberian Peninsula were very important for a clear distinction of species belonging to different subgenera [37]. In the light of this and with the preliminary genome size data, it seems that, as occurs in other genera, genome size and other cytological characters may also have high taxonomic significance within *Polygala*. Nevertheless, further studies with more species are needed to test this hypothesis. The genome size of *P. calcarea* has been previously estimated by Hanson et al. [26]. The value obtained by these authors (2C = 0.86 pg) is smaller than the value obtained in this work (2C = 0.98 pg). This 12% difference may be due to different reference standards (Hanson et al. [26] used *Vigna radiata* 'Bergen' with a 2C value of 1.06 pg while in our work *L. esculentum* was the reference standard), instruments and methodologies, as sample processing and lysis buffers (Hanson et al. [26] used Baranyi's buffer [38] while Tris·$MgCl_2$ was used in our work) [24,39,40].

With this work it was possible to easily evaluate the polysomaty of several organs of *P. vayredae* and *P. calcarea* using flow cytometry, contributing important information on the occurrence and pattern of endopolyploidy in new taxa. Additionally, the interesting genome size results highlighted the necessity of new and more extensive studies on this family, for which flow cytometry appears to be the ideal technique.

Table 2
Genome size estimations in *Polygala vayredae* and *P. calcarea*

Species	2C nuclear DNA content (pg)	1C genome size (Mbp)[a]	CV (%)		*n* (*R*)
			2C	4C	
Polygala vayredae	2.71 ± 0.046	1325	2.99	1.48	10 (3)
Polygala calcarea	0.98 ± 0.020	481	4.35	3.20	13 (2)

The values are given as mean and standard deviation of the nuclear DNA content in mass values (pg/2C) and as mean of the 1C genome size in number of base pairs (Mbp). For each species, the mean coefficient of variation (CV, %) of the 2C and 4C peaks, number of analysed individuals (*n*) and number of replicates (*R*) are also provided.

[a] 1 pg of DNA = 978 Mbp [33].

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