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

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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*

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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]