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ORIGINAL ARTICLE

Flow cytometric and karyological analyses of *Calendula* species from Iberian Peninsula

Sofia Nora · Sílvia Castro · João Loureiro · Ana Carla Gonçalves · Helena Oliveira · Mariana Castro · Conceição Santos · Paulo Silveira

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Abstract *Calendula* L. (Asteraceae) is a taxonomically and cytologically complex genus due to its high morphological and karyological variation. To gather consistent cytological information aiming to consolidate the existing knowledge, sustain the taxonomic revision of the genus and explore the evolutionary relationships among species, the genome size and chromosome number of the Iberian Peninsula representatives of this genus were assessed. The study included 11 *taxa* that occur in the Iberian Peninsula, one in Madeira and two from Morocco. Chromosome counts were made using the squash technique in root tips and flower buds, while nuclear DNA contents were assessed using propidium iodide flow cytometry. The following chromosome numbers are reported: $2n = 44$ for *C. arvensis*, $2n = 30$ for *C. tripterocarpa*, and $2n = 32$ for the remaining Iberian *taxa*. The genome size of *Calendula* species was assessed for the first time and ranged from 1.75 pg/2C in *C. maroccana* to 5.41 pg/2C in *C. arvensis*. Within the complex formed by *C. incana* and *C. suffruticosa*, a gradient of genome size values was obtained. Intraspecific variation in genome size was detected in some *taxa*. The obtained genome size values and their variation are discussed in the light of the theories proposed for the speciation of the genus, with events of hybridization, genome duplication and dysploidy being hypothesized to play a major role in the evolution of this genus.

Keywords *Calendula* · Chromosome numbers · Flow cytometry · Genome size · Iberian Peninsula · Karyology · Nuclear DNA content

Introduction

The genus *Calendula* L. belongs to Calenduleae Cass., one of the smallest tribes of the family Asteraceae (Bremer 1994). First described by Linnaeus in *Species Plantarum* (1753), the genus comprises 10–25 species, depending on the taxonomic treatment, distributed mainly in the Mediterranean basin (Norlindh 1946). It comprises annual and perennial plants, with sessile leaves, arranged alternately. The capitula are solitary with internal tubular yellow, orange, brown or violet-purple flowers, functionally male, and external yellow or orange ligulate female flowers. The achenes have no pappus and are heteromorphic (Nordenstam 1994). In *Calendula*, it is possible to observe, in the same capitulum, a continuum of fruit morphologies theoretically reflecting an evolution from the more ancestral forms at the periphery to more recent ones inside (Norlindh 1946). The production of more than one type of fruit per plant, known as heterocarpy, is a widespread reproductive strategy in Asteraceae (Zohary 1950).

Calendula is a taxonomically complex genus (Norlindh 1977; Heyn and Joel 1983), mainly because of the high levels of hybridization between different *taxa* (Lanza 1919;

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S. Nora
Department of Plant Biology and Ecology, Faculty of Pharmacy, University of Seville, 41012 Seville, Spain

S. Castro · J. Loureiro · M. Castro
CFE-Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, 3001-455 Coimbra, Portugal

A. C. Gonçalves · H. Oliveira · C. Santos · P. Silveira (✉)
Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal
e-mail: psilveira@ua.pt

Heyn and Joel 1983), and because of their large morphological variability, leading to the appearance of a wide range of intermediate forms, recognized by various names under different taxonomic categories (Heyn and Joel 1983). Several authors tried to study the taxonomy of the genus *Calendula* using different approaches (Norlindh 1946, 1977; Meusel and Ohle 1966; Heyn et al. 1974; Heyn and Joel 1983; Ohle 1974, 1975a, b). The works of Heyn et al. (1974) in annual *taxa* and Ohle (1974, 1975a, b) in perennial ones, represent the most recent and complete taxonomic reviews of the genus. Heyn et al. (1974) recognized five annual species: *C. tripterocarpa* Rupr. ($2n = 30$ chromosomes), *C. arvensis* L. ($2n = 44$), *C. stellata* Cav. (including *C. algeriensis* Boiss. and Reut.) ($2n = 14$), *C. palaestina* Boiss. and *C. pachysperma* Zoh (both with $2n = \pm 85$; the later also reported to have ca. 88 by Pazy 2000). *Calendula arvensis* is a synanthropic species with a wide distribution, whereas the remaining *taxa* are more geographically restricted. Ohle (1974, 1975a, b) changed the taxonomic category of some of the perennial species of the genus to previously described subspecies (Norlindh 1977). Essentially, these *taxa* were divided into three groups: two groups of the western Mediterranean region, *C. suffruticosa* and *C. incana*, both with $2n = 32$ chromosomes, and a third group which includes four distinct species with the same chromosome number ($2n = 18$): *C. maroccana* (Ball.) D.B.Jacks., *C. meuselii* Ohle, *C. eckerleinii* Ohle and *C. lanzae* Maire (the first three species are perennial and the last one is annual) (Ohle 1975a).

A large and increasing number of cytological characters, such as the number, morphology, and meiotic behavior of chromosomes, as well as the nuclear DNA content, have been used to circumscribe *taxa* and infer their relationships, being of pivotal importance to the systematic community, especially for resolving the taxonomy of complex groups (Kron et al. 2007). Indeed, the chromosome numbers have served as basis of various hypotheses about the evolution of the genus *Calendula* (Ehrendorfer 1970), and have been regarded as an important tool in the delimitation of several species, which in many cases were not so easily separated using morphoanatomic analyses. The basic chromosome numbers considered so far for this genus are seven ($2n = 14$, *C. stellata*), eight ($2n = 32$, most perennial *taxa* of northern Africa and southwestern Europe), nine ($2n = 18$, some perennials *taxa* of North Africa), 11 ($2n = 44$, *C. arvensis*) and 15 ($2n = 30$, *C. tripterocarpa*) (Darlington and Wylie 1955; Norlindh 1977, Heyn and Joel 1983; Nordenstam 1994). Heyn et al. (1974) raised interesting hypotheses regarding the ploidy level in *Calendula*. These authors reported, for the first time, the chromosome number of *C. palaestina* and *C. pachysperma* as $2n = \pm 85$, considering them as autopolyploids of *C. arvensis* ($2n = 44$). In addition, *C. arvensis* was proposed to be a tetraploid hybrid of two *taxa* with the chromosome numbers of 15 and 7. Finally, it was confirmed that the majority of the *taxa* with low chromosome numbers, both annual and perennial, occurred in the southern part of the Mediterranean, supporting the idea proposed by Norlindh (1946) that the primary center of evolution of the genus is located in this region (for more details, see Heyn and Joel 1983).

Despite the importance of these classical cytological studies, the employed techniques are known to be time-consuming and particularly difficult in groups with small and numerous chromosomes, such as the annual *Calendula* spp. The advent of flow cytometry (FCM) has opened the possibility to alter this scenario, being now feasible to obtain reliable estimations of nuclear DNA content (and thus, ploidy level and genome size) with ease and in a short period of time. Numerous taxonomic and biosystematic studies have already shown the usefulness of this character (e.g., Loureiro et al. 2007a; Temsch and Greilhuber 2010; Campos et al. 2011), being the use of FCM increasingly rising in recent times (Kron et al. 2007). However, regardless of the increasing importance of nuclear DNA content in biosystematic studies, there are no genome size estimations available for the genus *Calendula* so far.

The objectives of this study were: (1) to estimate, for the first time, the nuclear DNA content of the *Calendula* spp. from Iberian Peninsula using FCM; (2) to determine the chromosome numbers of the different *taxa* from this region based on reliable taxonomic determinations, and compare the obtained results with data from the literature; (3) to evaluate whether the nuclear DNA content data support the splitting of the wild Iberian perennial *Calendula* in two groups, *C. incana* and *suffruticosa* as proposed by Ohle (1974); (4) to assess the role of genome size in providing insights on the relationships and evolution of *Calendula* species (Heyn and Joel 1983). The study included the 11 *taxa* that occur in the Iberian Peninsula, one from Madeira, *C. incana* subsp. *maderensis*, and two additional *taxa* from Morocco, *C. stellata* and *C. maroccana*.

Materials and methods

Plant material

Individual plants, leaves and seed samples from 11 *taxa* of *Calendula* were collected from 31 field locations in the Iberian Peninsula, one from Madeira Archipelago and two additional *taxa*/populations from Morocco (see Online Resource 1). For problematic *taxa*, whenever possible, field collections included type populations or localities mentioned by Ohle (1974) (Online Resource 1). Seeds were soaked overnight in running water, placed in darkness, in

Petri dishes with moistened filter paper and, after approximately one week at 4 °C, they were transferred to 25 °C to germinate. Field-collected and seed-germinated plants were potted and maintained in homogeneous conditions (20 ± 2 °C, with a light intensity of 60 ± 5 mol m^{-2} s^{-1}) and further used for chromosome counts and nuclear DNA content estimations using flow cytometry (FCM). Voucher specimens were collected and kept in the Herbarium of the University of Aveiro (AVE). In this study, we followed the taxonomic nomenclature proposed by Ohle (1974, 1975a, b) for the perennial *taxa* and by Heyn et al. (1974) for the annual *taxa*.

Chromosome counts

Somatic chromosome counts were obtained in mitotic metaphase spreads using healthy root tips of seedlings and adult plants, whereas gametic counts were determined at meiosis using the floral buds (floral buds were collected in different early stages of development to assure that microspore mother cells were captured at a stage prior to the formation of the pollen grains). In both cases, the methodology described in Aparicio (1989) was followed. Briefly, healthy root tips were pre-treated for 12 h with cold water (4 °C) (Norlindh 1963), and were then, fixed at room temperature in a solution of ethanol/glacial acetic acid (3:1) for 24 h; flower buds were fixed directly after harvest. Fixed material was stored in 70 % ethanol until analysis. Plant material was stained with alcoholic hydrochloric acid-carmine for a minimum of 24 h. The microscopic slides were prepared using the squash method. Images of chromosome spreads were acquired with a Leica digital camera DC200 (Gmbtt Leica Microsystems, Wetzlar, Germany) incorporated in a Nikon Eclipse 80i microscope (Nikon Instruments, NY, USA), and processed using the Leica IM1000 v.1.1 software (Leica Microsystems AG, Heerbrugg, Switzerland). The genome size of the plants used for chromosome counts was assessed following the methodology described below and enabled the assignment of a chromosome number to genome size estimations.

Karyological information of the studied species was also compiled from several indices of chromosome numbers (Darlington and Wylie 1955; Bolkhovskikh et al. 1969; Goldblatt and Johnson 1979; Martín Ciudad 1991; Pastor Díaz 1997), previous publications (54 articles, mainly composed by regional records of chromosomal numbers), and web databases (Index to Plant Chromosome Numbers—Tropicos®, http://www.tropicos.org).

Nuclear DNA content estimations

Nuclear DNA content was estimated by FCM using the methodology described by Loureiro et al. (2007a). Briefly, nuclear suspensions were prepared by chopping 50 mg of leaf tissue of *Calendula* spp. and 50 mg of internal standard leaves (*Pisum sativum* 'Ctirad'), with a razor blade in a glass Petri dish containing 1 mL of WPB isolation buffer (200 mM Tris HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA $Na_2 \cdot 2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7.5; Loureiro et al. 2007b). The nuclear solution was then filtered through a nylon net of 50 µm, and 50 µg mL^{-1} of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 µg mL^{-1} of RNAse (Fluka, Buchs, Switzerland) were added to the sample, to stain nuclear DNA and prevent staining of double-stranded RNA, respectively. Samples were analyzed within a 10-min period on a Beckman–Coulter EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL, USA) equipped with a 25-mW air-cooled argon laser and operating at 488 nm.

Results were acquired using the SYSTEM II™ (v. 2.5) software in the form of five graphics: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS) in logarithmic (log) scales; FL vs. time; FL vs. fluorescence pulse height; FL vs. SS in log scale. In most samples, in the latter graphic, a polygonal region was defined to include only intact nuclei; this region was used to gate all the other graphics. For each sample, at least 5,000 particles were analyzed. As a quality control, nuclear DNA content estimates were only considered when the coefficient of variation of G_0/G_1 peaks (CV_{peak}) were below 5 %. Samples with higher CV_{peak} values were discarded and a new sample was prepared. In some cases where intraspecific variation was suspected, simultaneous analysis of such samples was performed, to confirm (the presence of double G_0/G_1 peaks) or reject (single G_0/G_1 peaks) its occurrence (Fig. 2f).

The holoploid genome size in pg (2C; sensu Greilhuber et al. 2005) for each *taxa* was calculated by multiplying the known nuclear DNA content of the reference standard plant (*Pisum sativum*, 2C = 9.09 pg of DNA, Doležel et al. 1998) by the ratio between the mean fluorescent intensities of the G_0/G_1 peaks of the *Calendula* spp. and the reference standard. The monoploid genome size (1Cx; sensu Greilhuber et al. 2005) in Mbp (1 pg = 978 Mbp, Doležel et al. 2003) was also calculated for all species by dividing their holoploid genome size (2C) by the supposed ploidy level of each *taxa*.

Statistical analyses

Descriptive statistics were calculated for each *taxa* studied namely, mean, standard deviation (SD), coefficient of variation (CV), and minimum and maximum values of the holoploid genome size (2C, pg). Differences in holoploid genome size among all the *taxa* studied were tested using a Kruskal–Wallis ANOVA on ranks (because the data were

Table 1 Observed and reported chromosome numbers in the *Calendula* taxa of the Iberian Peninsula

Taxa	Chromosome number				References
	Observed		Reported		
	n	2*n*	*n*	2*n*	
C. arvensis L.	–	44	22[a]	14[b], 18[c], 36[d], 42[e], 44[f], 46[g]	[a]Heyn and Joel (1983); Aparicio (1989) [b,c]Humphries *et al.* (1978) [d]Negodi (1936); Janaki-Ammal and Sobti (1962); Pawlowski and Jasiewicz (1971); Malallah and Brown (1999) [e]Borgen (1974); Löve and Kjellqvist (1974); Albers and Pröbsting (1998) [f]Meusel and Ohle (1966); Dahlgren et al. (1971); Fernandes and Queirós (1971a); Nordenstam (1972); Queirós (1973); Heyn et al. (1974); Marchi et al. (1974); Powell et al. (1974); Scrugli (1974); Van Loon (1974); Borgen (1975); Diaz Diaz Linfante et al. (1992); Oberprieler and Vogt (1993); Malallah and Brown (1999); Vogt and Aparício (1999); Baltisberger and Widmer (2006); Vogt and Oberprieler (2008) [g]Rashid (1974)
C. arvensis ssp. *macroptera* Rouy	–	–	–	44	Gallego (1983)
C. incana Willd. ssp. *incana*	–	–	16[a]	18[b], 32[c]	[a]Heyn and Joel (1983); Ruiz de Clavijo (1989) [b]Meusel and Ohle (1966) [c]Ohle (1974); Vogt and Oberprieler (2008)
C. incana ssp. *algarbiensis* (Boiss.) Ohle	–	–	–	32	Ohle (1974); Strother and Watson (1997)
C. incana ssp. *algarbiensis* var. *cinerea* Ohle	–	–	–	32	Ohle (1974)
C. incana subsp. *maderensis* (DC.) Ohle §	–	–	–	32	Meusel and Ohle (1966); Ohle (1974); Press and Short (1994)
C. incana ssp. *microphylla* (Lange) Ohle	–	32	–	20[a], 32[b]	[a]Fernandes and Queirós (1971b) [b]Mesquita Rodrigues (1953); Meusel and Ohle (1966); Fernandes and Queirós (1971a); Queirós (1973), Ohle (1974)
C. maroccana Ball ssp. *maroccana**	–	–	–	18	Oberprieler and Vogt (1993); Ohle (1975a, b); Valdés and Parra (1997)
C. officinalis L.	–	32	16[a]	14[b], 28[c], 32[d]	[a]Mehra et al. (1965); Gupta (1969); Gupta et al. (1972); Mehra and Remanadan (1976); Heyn and Joel (1983); Mathew and Mathew (1988) [b]Carr et al. (1999) [c]Negodi (1936); Májovský (1978); Murin (1993) [d]Weddle (1941); Janaki-Ammal and Sobti (1962); Meusel and Ohle (1966); Ohle (1974); Baltisberger and Huber (1987); Gupta and Gill (1989); Pogan et al. (1989); Pogan and Wcislo (1990); Murin (1997)
C. suffruticosa ssp. *carbonelli* Ohle	–	–	–	32	Ohle (1974)
C. suffruticosa ssp. *greuteri* Ohle	–	–	–	32	Ohle (1974)
C. suffruticosa ssp. *lusitanica* (Boiss.) Ohle	16	32	16[a]	32[b]	[a]Talavera (1979) [b]Meusel and Ohle (1966); Fernandes and Queirós (1971a); Ohle (1974); Meikle (1976)
C. tripterocarpa Rupr.	–	30	15[a, e]	30[b], 30 + 2B[c], 54[d]	[a]Aparicio (1989) [b]Meusel and Ohle (1966); Heyn et al. (1974), Diaz Linfante et al. (1992) [c]Oberprieler and Vogt (1993); Vogt and Oberprieler (2008) [d]Dalgaard (1986) [e]Heyn and Joel (1983)

Table 1 continued

Taxa	Chromosome number				References
	Observed		Reported		
	n	2*n*	*n*	2*n*	
C. stellata Cav.*	–	–	7[a]	14[b]	[a]Aparicio (1989); Ruiz de Clavijo (1991); Talavera et al. (1984) [b]Heyn et al. (1974); Vogt and Oberprieler (2008)

* Including two *taxa* from Morocco and one § from Madeira archipelago

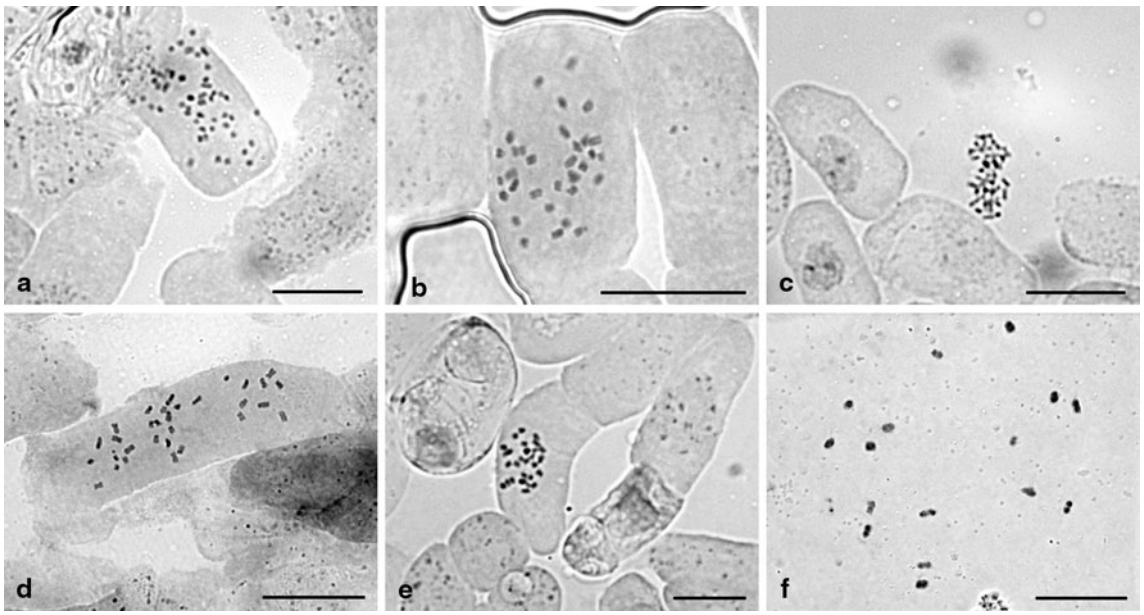

Fig. 1 Chromosomes in *Calendula* spp. **a** Metaphase plate in *Calendula arvensis*, $2n = 44$; **b** metaphase plate in *Calendula officinalis*, $2n = 32$; **c** metaphase plate in *Calendula incana* ssp. *microphylla*, $2n = 32$; **d** metaphase plate in *Calendula suffruticosa* ssp. *lusitanica*, $2n = 32$; **e** metaphase plate in *Calendula tripterocarpa*, $2n = 30$; **f** Cell of *Calendula suffruticosa* ssp. *lusitanica* in diakinesis ($n = 16$). *Scale bars*: **a**–**e** 50 µm; **f** 10 µm

not homoscedastic, even after transformation), followed by a Dunn's multiple comparison test to assess which groups were different. The same tests were applied to *C. incana* and *C. suffruticosa taxa*, to evaluate statistically the differences in genome size among the taxa of these two closely related entities. The mean holoploid genome size of each *taxa* was also correlated with chromosome number obtained and/or reported for the *taxa* using a Pearson correlation.

Results

Chromosome counts

The chromosome numbers determined in this study are presented in Table 1 and illustrated in Fig. 1. The observed chromosome counts were in agreement with the previously reported estimations (Table 1). The number of chromosomes observed in the accessions belonging to *C. incana* ssp. *microphylla*, *C. suffruticosa* ssp. *lusitanica* and *C. officinalis* was $2n = 32$ chromosomes (Fig. 1b, d), whereas for *C. arvensis* and *C. tripterocarpa*, $2n = 44$ and $2n = 30$ chromosomes were observed, respectively (Fig. 1a, e). The value obtained for *C. suffruticosa* ssp. *lusitanica* was further supported by the counting of $n = 16$ chromosomes in the flower buds (Fig. 1f). No B-chromosomes were observed in the studied *taxa*.

Nuclear DNA content estimations

The 2C nuclear DNA content of the Iberian Peninsula *Calendula taxa* was determined, for the first time, using flow cytometry (Table 2). Fluorescence histograms of relative nuclear DNA content showed clear G_0/G_1 peaks with CV_{peak} values lower than 3.40 % (Fig. 2). *Calendula* species holoploid genome sizes (2C) ranged from 1.75 pg/2C in *C. maroccana* to 5.41 pg/2C in *C. arvensis*. Statistically significant differences in genome size were obtained when

Table 2 Nuclear DNA content estimations in *Calendula* spp. from Iberian Peninsula (*including two *taxa* from Morocco)

Taxon	Habit	Ploidy level	Genome size (2C, pg)				Genome size (1C, Mbp)	*n* pop.	*n* Total
			Mean ± SD	Min.	Max.	CV (%)			
C. arvensis	Annual	$2n = 4x = 44$	5.41 ± 0.11^{a}	5.20	5.64	2.05	1323	4	16
C. incana ssp. *incana*	Perennial	$2n = 4x = 32$	3.24 ± 0.07^{b}	3.10	3.34	2.16	792	2	10
C. incana ssp. *algarbiensis* var. *algarbiensis*	Perennial	$2n = 4x = 32$	3.05 ± 0.07^{bc}	2.97	3.19	2.13	746	3	15
C. incana ssp. *algarbiensis* var. *cinerea*	Perennial	$2n = 4x = 32$	3.18 ± 0.16^{bc}	3.03	3.38	5.00	778	1	7
C. incana ssp. *maderensis*	Perennial	$2n = 4x = 32$	3.21 ± 0.06^{cd}	3.10	3.29	1.87	785	1	7
C. incana ssp. *microphylla*	Perennial	$2n = 4x = 32$	3.34 ± 0.09^{bc}	3.17	3.57	2.70	817	4	24
C. maroccana ssp. *maroccana**	Perennial	$2n = 2x = 18$	1.75 ± 0.05^{e}	1.70	1.80	2.57	856	1	5
C. officinalis	Annual/ Perennial	$2n = 4x = 32$	2.97 ± 0.08^{b}	2.89	3.15	2.59	726	3	14
C. suffruticosa ssp. *carbonellii*	Perennial	$2n = 4x = 32$	3.21 ± 0.11^{bc}	3.04	3.42	3.29	785	2	17
C. suffruticosa ssp. *greuteri*	Annual/ Perennial	$2n = 4x = 32$	3.42 ± 0.13^{cd}	3.15	3.63	3.72	836	8	49
C. suffruticosa ssp. *lusitanica*	Perennial	$2n = 4x = 32$	3.29 ± 0.11^{cd}	3.02	3.41	3.03	804	3	18
C. tripterocarpa	Annual	$2n = 2x = 30$	3.47 ± 0.08^{d}	3.35	3.62	2.24	1696	1	15
*C. stellata**	Annual	$2n = 2x = 14$	2.05 ± 0.06^{e}	1.98	2.16	2.91	1002	1	7

Ploidy level and mean (±standard deviation, SD), minimum and maximum of the holoploid genome size (2C, pg) is given for each *taxa* studied. The coefficient variation (CV, %) of the holoploid genome size, the monoploid genome size (1Cx) in Mbp (ratio: 1 pg DNA = 978 Mbp; Doležel et al. 2003) and the number populations (*n* pop.) and individuals (*n* total) analysed are also provided. Different letters reveal statistically significant differences at $P \leq 0.05$ according to Dunn's multiple comparison test

comparing all the *taxa* studied ($H_{12} = 158.35$, $P \leq 0.001$), with *C. arvensis* having significantly higher genome size than the other *taxa*, while *C. maroccana* and *C. stellata* presented significantly lower genome sizes. Considering the number of chromosomes, the genome size estimation obtained for *C. stellata* was unexpectedly higher than the one obtained for *C. maroccana* (Table 2). In addition, the mean values of genome size were positive and significantly correlated with the number of chromosome ($R = 0.913$, $P < 0.001$). The analysis of genome size variation in the homoploid *taxa* (i.e., *taxa* with $2n = 32$) revealed that *C. officinalis*, the only cultivated species, presented lower genome size (2.97 pg/2C) than the remaining *taxa* (Table 2).

According with the literature, most of the *taxa* analysed were interpreted as tetraploids, with the exception of *C. tripterocarpa*, *C. maroccana* and *C. stellata* that were assumed to be diploids. Taking this into consideration, the analysis of the monoploid genome size (1Cx; 2) revealed that the annuals *C. tripterocarpa* and *C. arvensis* had the higher values for this trait, coinciding also with the highest basic chromosome numbers ($n = 15$ and 11, respectively). *Calendula stellata* had a surprisingly high monoploid genome size considering that it has the lowest basic chromosome number ($n = 7$). The remaining *taxa* were fairly homogeneous regarding their monoploid genome size (Table 2).

Statistically significant differences in genome size were also observed among the analysed *taxa* of the closely related *C. incana* and *C. suffruticosa* groups ($H_7 = 77.90$, $P \leq 0.001$; Fig. 3). Overall, subspecies of *C. incana* presented slightly lower genome size values than those of *C. suffruticosa*; still, a continuum in genome size values ranging from 3.05 pg/2C in *C. incana* ssp. *algarbiensis* var. *algarbiensis* to 3.42 pg/2C in *C. suffruticosa* ssp. *greuteri* was observed (Table 2; Fig. 3), with two subspecies (*C. incana* ssp. *microphylla* with 3.34 pg/2C, and *C. suffruticosa* ssp. *carbonellii* with 3.21 pg/2C), presenting genome size values falling into the range of the opposite species.

With some exceptions, the majority of *taxa* presented a high homogeneity in the genome size estimates among populations and individual plants of each *taxon* (genome size CV values below 3 %). However, in *C. incana* ssp. *algarbiensis* var. *cinerea* and in the three subspecies of *C. suffruticosa*, a higher heterogeneity in genome size was found, with CV values surpassing the 3 % threshold, reflecting the possible occurrence of intraspecific variation in genome size in these *taxa*. The occurrence of this phenomenon was further confirmed after simultaneous analysis of samples with different genome sizes (Fig. 2f). In the example presented, the difference in mean fluorescence detected (9.05 %) between the individuals corresponds to the difference in its genome size (3.07–3.36 pg/2C).

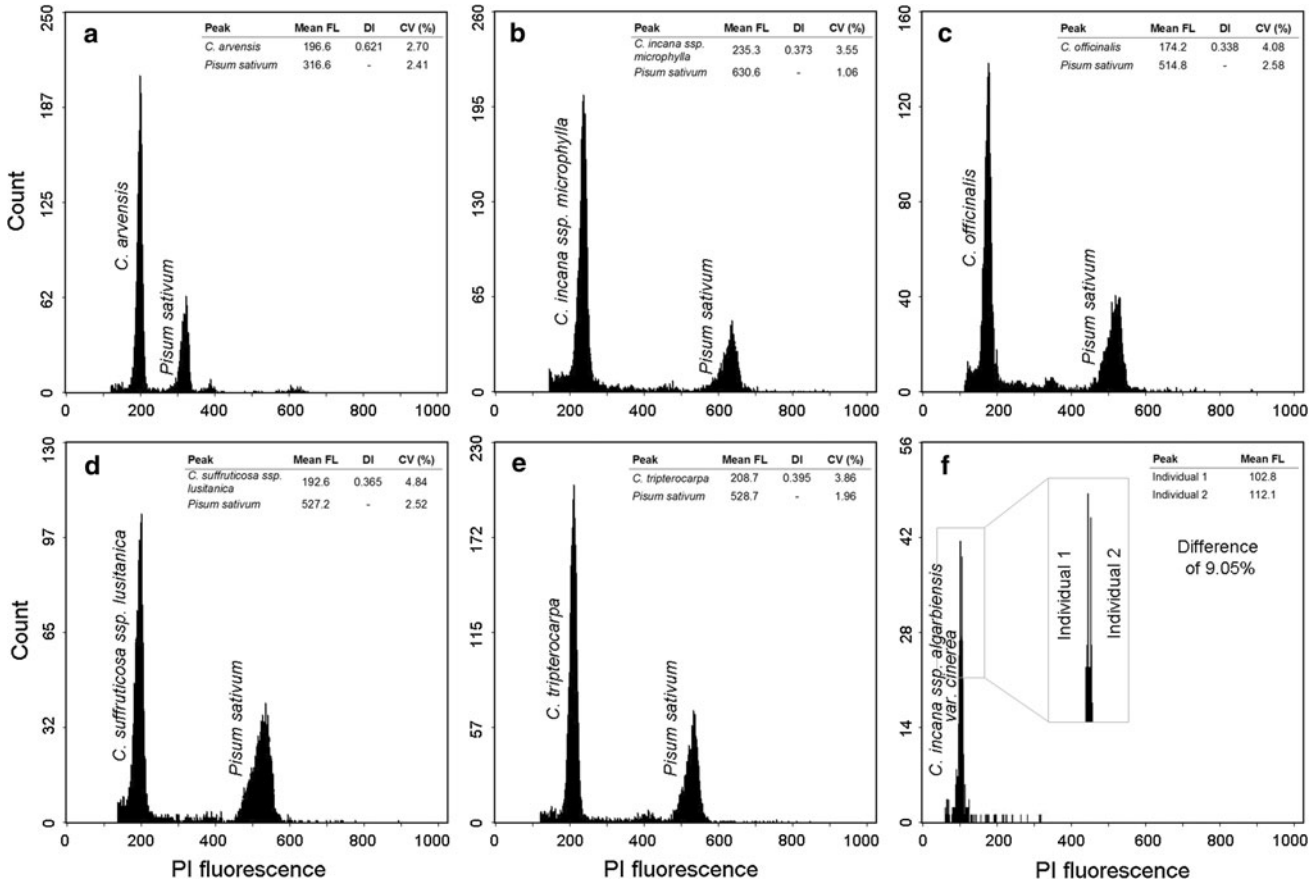

Fig. 2 Flow cytometric histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Pisum sativum* 'Ctirad' (2C = 9.09 pg of DNA, the internal reference standard) and *Calendula* spp.: **a** *C. arvensis* ($2n = 44$), **b** *C. incana* ssp. *microphylla* ($2n = 32$), **c** *C. officinalis* ($2n = 32$), **d** *C. suffruticosa* ssp. *lusitanica* ($2n = 32$), **e** *C. tripterocarpa* ($2n = 30$), and **f** simultaneous analysis of two individuals of *C. incana* ssp. *algarbiensis* var. *cinerea* with different 2C values (3.07 and 3.36 pg/2C, respectively). The histograms show G_0/G_1 peaks of the sample and of the internal standard. The *inset tables* provide the mean channel number (mean FL), DNA index (*DI* mean channel number of sample/mean channel number of reference standard) and coefficient of variation of each peak (CV, %)

Discussion

The amount of DNA per chromosome set is known to be a fairly constant characteristic of a species. Therefore, in addition to the number of chromosomes, which are since long regarded as an important tool for delimiting species, in the past two decades an increasing interest on genome size studies and its significance has been observed, with many studies aiming at using genome size as a taxonomic marker (Kron et al. 2007). Despite this increase, the complex genus *Calendula* has been completely neglected in the literature and the genome size values presented in this study are the first estimates for the genus.

Cytologically, *Calendula* is considered a complex genus where chromosome losses, dysploidy, hybridization events and genome duplications are proposed to be in the genesis of the several basic chromosome numbers reported (7, 8, 9, 11 and 15; Nordenstam and Källersjö 2009), resulting in the wide diversity of chromosome numbers observed in such a small genus (Heyn and Joel 1983). In the Iberian *Calendula* species, $2n = 32$ was the most commonly observed chromosome number, with only *C. arvensis* and *C. tripterocarpa* possessing different chromosome numbers ($2n = 44$ and $2n = 30$, respectively). These chromosome counts are in agreement with the majority of the values cited in the bibliography (reviewed in Table 1). The two Moroccan species had lower chromosome numbers and ploidy level than the Iberian *taxa* (Heyn et al. 1974; Oberprieler and Vogt 1993; Ohle 1975a; Valdés and Parra 1997; Vogt and Oberprieler 2008).

Based on the reproductive relationships and chromosome numbers of annual species, Heyn and Joel (1983) proposed a hypothesis for the speciation process in the genus *Calendula*, over which the genome size values obtained in this study were overlapped (Fig. 4). In this hypothesis, *C. maroccana* and *C. stellata* that belong to the region considered as the primary center of evolution of *Calendula* (Norlindh 1946) played a central role in the

origin of several *taxa*, and hybridization and polyploidization events were proposed as the main mechanisms giving rise to new entities. First, *C. stellata* was proposed to have originated from *C. maroccana* after several steps of chromosome reductions; however, the results of genome size obtained in the present study do not support this step, as the genome size of *C. stellata* is higher than that of *C. maroccana*. For the western Mediterranean group, including *C. suffruticosa*, *C. incana* and *C. officinalis*, two major paths were proposed. Heyn and Joel (1983) hypothesized that chromosome losses by *C. maroccana* originated an entity with $2n = 16$ chromosomes (assumed genome size of 1.56 pg/2C; value estimated assuming that all chromosomes possess the same amount of DNA, which may not be entirely true) that subsequently suffered a genome-duplication event, originating an entity with $2n = 32$ chromosomes. Our results of genome size do not fully support this hypothesis as a relatively low genome size was obtained for the hypothesized polyploid (3.12 pg/2C). Another hypothesis based on morphological similarities is that the perennial *taxa* from the western Mediterranean region could have resulted from hybridization between *taxa* with different chromosome numbers, such as $2n = 14$ (e.g., *C. stellata*) and $2n = 18$ chromosomes (e.g., *C. maroccana*, *C. eckerleinii*), followed by chromosome doubling of the hybrid with $2n = 16$ (Ohle 1974, 1975a, b). Using the *taxa* studied, the estimates of genome size do not support this hypothesis as a genome size higher than all the obtained estimates would have been obtained after genome duplication (3.80 pg/2C).

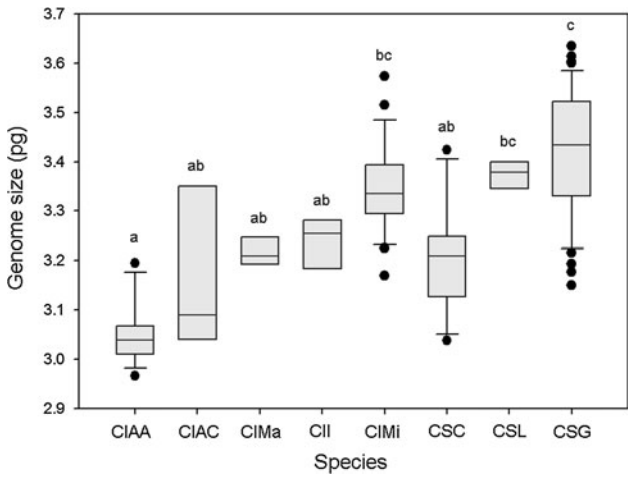

Fig. 3 Nuclear DNA content (2C values) of *C. incana* and *C. suffruticosa* subspecies: *C. incana* ssp. *algarbiensis* var. *algarbiensis* (CIAA), *C. incana* ssp. *algarbiensis* var. *cinerea* (CIAC), *C. incana* ssp. *maderensis* (CIMa), *C. incana* ssp. *incana* (CII), *C. incana* ssp. *microphylla* (CIMi), *C. suffruticosa* ssp. *carbonellii* (CSC), *C. suffruticosa* ssp. *lusitanica* (CSL) and *C. suffruticosa* ssp. *greuteri* (CSG). The values are distributed over four areas of equal frequency (quartiles), the 25th and 75th percentiles are represented by a *box*, the median by a line, the *outer lines* indicate the minimum and maximum values and the points represent outliers. Different *letters* reveal statistically significant differences at $P \leq 0.05$ according to Dunn's multiple comparison test

Considering all these, another important mechanism of genome evolution that has been neglected so far, dysploidy, may be hypothesized to be involved in the origin of these taxa. Dysploidy is a structural change in the number of chromosomes through a re-arrangement of chromatin and loss or gain of a centromere without necessarily changing the amount of chromatin in the karyotype. Decreasing dysploidy is a common feature in Asteraceae and is considered an active evolutionary mechanism in the family (Garcia-Jacas et al. 1996; Semple and Watanable

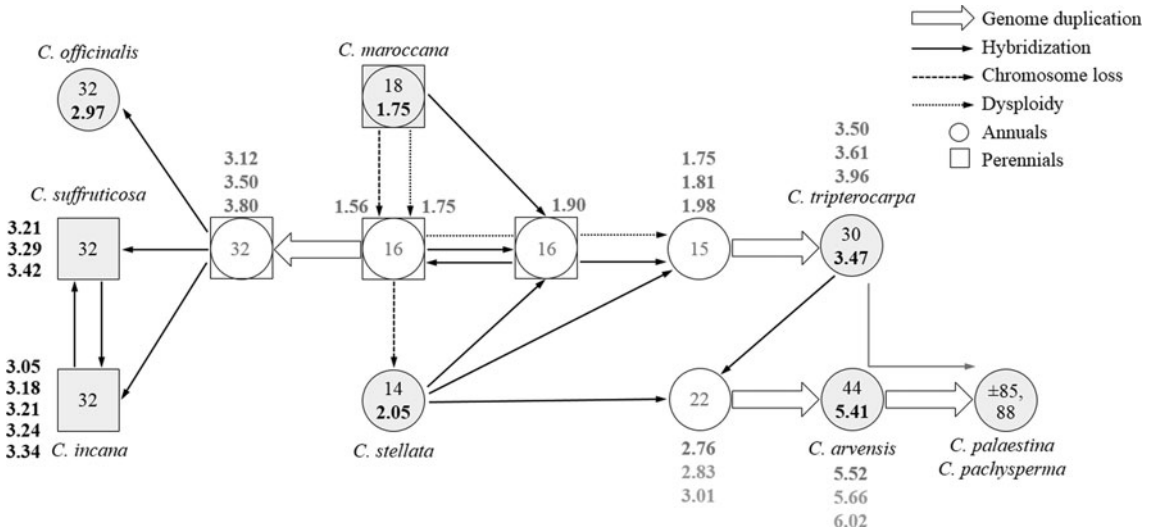

Fig. 4 Proposed scheme of speciation in *Calendula* based in chromosome numbers (entire numbers) adapted from Heyn and Joel (1983): *grey* values represent hypothetical genome sizes of ancestral species, while *black* values represent genome size estimations of current species

2009). In addition, it is frequently proposed as a mechanism behind series of basic chromosome numbers (Semple and Watanable 2009). Indeed, dysploidy has been proposed by Nordenstam (1994) to interpret the diversity of basic chromosome numbers in *Calendula*. If one considers a descending dysploidy event from *C. maroccana* (resulting in a $2n = 16$ entity with the same genome size of the origin species) and subsequent genome duplication, a theoretical genome size of 3.5 pg/2C would have been obtained; this value is actually in line with the genome size estimations obtained for some taxa of *C. suffruticosa* and *C. incana*; then, different levels of genome up and downsizing that are frequently associated with polyploidization and hybridization events (Leitch and Bennett 2004) may have originated the diversity of genome sizes that were obtained. Still, in all these hypotheses, chromosome gains/losses may have occurred, the origin *taxa* may have been different from the ones assumed in here and in the literature, or even a combination of other pathways and subsequent duplication and hybridizations may have occurred.

Calendula tripterocarpa was suggested to have originated after hybridization between *C. stellata* and: (1) *C. maroccana*, *C. stellata* hybrid, or (2) *C. maroccana* after the loss of two chromosomes. The subsequent genome duplication of these hybrids would have theoretically result in an entity with genome size values of 3.96 and 3.61 pg/2C, respectively, which are relatively higher than the value obtained for *C. tripterocarpa* (Heyn and Joel 1983). Again, dysploidy could also be considered; if that was the case, genome duplication of an individual originated from the hybridization between *C. stellata* and a $2n = 16$ entity with the same genome size of *C. maroccana* (through dysploidy) would have a theoretical genome size of 3.50, which is close to the estimations that were obtained for *C. tripterocarpa*. Finally, it was hypothesised that *C. arvensis* results from a cross between *C. stellata* and *C. tripterocarpa*, and subsequent duplication of the genome. The theoretical genome obtained using the estimations for *C. tripterocarpa* and *C. stellata* agrees with this hypothesis. The higher number of chromosomes observed in *C. palaestina* and *C. pachysperma* suggest an autopolyploid origin from *C. arvensis* (Heyn et al. 1974), but this hypothesis remains to be confirmed using genome size data.

The nuclear DNA content and chromosome number are usually positively correlated (Srivastava and Lavania 1991; Soliman 2003) and this general trend was also observed in *Calendula*. Still, *C. tripterocarpa* and *C. stellata* had higher values of genome size than expected in comparison with the other *taxa* and considering their chromosome numbers. This may suggest a distinct evolutionary line, as suggested above. *Calendula officinalis* also get aside from the pattern, having a relatively lower genome size than its homoploid counterparts. This decrease in genome size might be due to cultivation processes, as reported for several other cultivated species (e.g., Garcia et al. 2006; Nagato et al. 1981).

Infra-generic variation in genome size among homoploid species is a common feature in plants (Bennett and Leitch 2011), and thus, in some groups, genome size can be used as an additional marker to separate homoploid taxa (for a review see Loureiro et al. 2010). Within *Calendula*, the classification of the homoploid group that includes *C. suffruticosa* and *C. incana* ($2n = 32$ chromosomes) is rather complex and has for long time generated divergent interpretations. While some authors regard them as two distinct species containing several subspecies (Ohle 1974, 1975a, b, taxonomic nomenclature used in this study), others joined the two *taxa* in a single polymorphic species under the name of *C. suffruticosa* (e.g., Meikle 1976). The analysis of genome size detected overall differences among the taxa, with *C. suffruticosa* subspecies having, in general, higher nuclear DNA contents than those of *C. incana*. However, a clear gradient between the two species was observed, with intermediate values of genome size being detected in some subspecies (*C. incana* spp. *microphylla* and *C. suffruticosa* spp. *carbonellii*). This might be due to hybridization events, which are considered to be frequent in the genus (Weddle 1941; Janaki-Ammal and Sobti 1962; Heyn and Joel 1983). Thus, genome size fails to completely assist the delimitation of the two species and, considering the continuum in the estimations, it may actually support their unification in one single species.

The analysis of several individuals per population and several populations per *taxa* enabled to detect some cases of intraspecific variation in genome size. Various molecular mechanisms (e.g., duplications, deletions, chromosomal polymorphisms, the presence of B-chromosomes and/or the presence of repetitive sequences) have been proposed to be involved in this phenomenon, with both decreases and increases in nuclear DNA content having already been detected. Despite being considered a controversial topic for a long time, recent reports that followed best practices of FCM confirmed the existence of this phenomenon and correlated it with several ecological and environmental parameters (e.g., Šmarda and Bureš 2010; Cullis 2005; Turpeinen et al. 1999). In this study, intraspecific variation was present in *C. incana* ssp. *algarbiensis* var. *cinerea* and in the three subspecies of *C. suffruticosa*. Though not detected in the chromosome counts, the occurrence of satellite chromosomes, already reported for the genus *Calendula* (Strother and Watson 1997; Oberprieler and Vogt 1993; Vogt and Oberprieler 2008), may be among the factors responsible for the intraspecific genome size variation that was observed. Still, despite all the best practices of FCM analyses, the negative effect of cytosolic

compounds (considering that *Calendula* spp. are also phytochemically complex) on fluorescence (Loureiro et al. 2006) cannot be completely discarded.

In conclusion, this work contributed with important basic scientific knowledge on genome size and chromosome counts in the cytologically complex genus of *Calendula*, consolidating the existing knowledge on the taxonomic relationships, reviewing the hypotheses for the speciation process and providing important background information for subsequent studies. Indeed, regarding genome size evolution, several doors were opened, with increasing evidences for the occurrence of dysploidy and intraspecific variation in genome size.

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