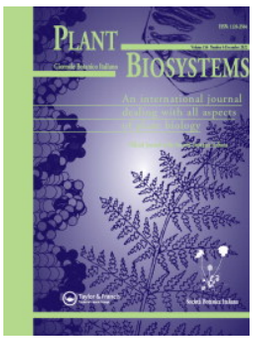

Taylor & Francis
Taylor & Francis Group

Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology

Official Journal of the Societa Botanica Italiana

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tplb20

Contribution to the knowledge of genome size variation in *Calendula* L. (Asteraceae) with special focus on the SW Mediterranean region

Ana Carla Gonçalves, Helena Oliveira, João Loureiro, Sílvia Castro, Maria Eduarda Fidalgo, Teresa Ribeiro, Ahmed Ouhammoud, Rachid Amirouche, Leonor Morais-Cecílio, Conceição Santos & Paulo Silveira

To cite this article: Ana Carla Gonçalves, Helena Oliveira, João Loureiro, Sílvia Castro, Maria Eduarda Fidalgo, Teresa Ribeiro, Ahmed Ouhammoud, Rachid Amirouche, Leonor Morais-Cecílio, Conceição Santos & Paulo Silveira (2023): Contribution to the knowledge of genome size variation in *Calendula* L. (Asteraceae) with special focus on the SW Mediterranean region, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, DOI: 10.1080/11263504.2023.2165551

To link to this article: https://doi.org/10.1080/11263504.2023.2165551

Published online: 19 Jan 2023.

PLANT BIOSYSTEMS - AN INTERNATIONAL JOURNAL DEALING WITH ALL ASPECTS OF PLANT BIOLOGY
https://doi.org/10.1080/11263504.2023.2165551

Contribution to the knowledge of genome size variation in *Calendula* L. (Asteraceae) with special focus on the SW Mediterranean region

Ana Carla Gonçalves[a], Helena Oliveira[a], João Loureiro[b], Sílvia Castro[b], Maria Eduarda Fidalgo[c], Teresa Ribeiro[c], Ahmed Ouhammoud[d], Rachid Amirouche[e], Leonor Morais-Cecílio[c], Conceição Santos[f] and Paulo Silveira[a]

[a]Departamento de Biologia e CESAM, Universidade de Aveiro, Aveiro, Portugal; [b]Departamento de Ciências da Vida e Centro de Ecologia Funcional, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Calçada Martim de Freitas, Coimbra, Portugal; [c]Linking Landscape, Environment, Agriculture and Food, Associated Laboratory TERRA Instituto Superior de Agronomia, University of Lisbon, Tapada da Ajuda, Lisboa, Portugal; [d]Herbier Régional 'MARK', Laboratoire d'Ecologie et Environnement, Faculté des Science Semlalia, Université Cadi Ayyad, Marrakech, Maroc; [e]Laboratoire de Biologie et Physiologie des Organismes, Université des Sciences et de la Technologie Houari Boumediene, USTHB, Bab-Ezzouar, Alger, Algeria; [f]Departamento de Biologia & LAQV/REQUIMTE, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

ABSTRACT

Calendula is one of the most taxonomically complex genera within the Asteraceae family due to hybridization, polyploidization events, and production of a highly variable morphology of the achenes. Considering the complexity of *Calendula*, this study was conducted to extend the understanding of the relationships between SW Mediterranean taxa, and assess the relationships between genome size and chromosome number, ploidy level and life cycle in 77 populations covering 14 taxa. Genome size estimations are provided for the first time for five species and for three putative new undescribed species. Mean 2C values differed up to 6-fold among different euploid species (from 1.37 pg in diploid to 8.26 pg in octoploid populations). 1Cx-values varied 2.07-fold (between 0.68 pg and 1.41 pg). Mean 1Cx genome size revealed significant differences between different ploidy levels. Genome size variation is a significant factor for explaining the relationships within *Calendula*, and individuals not fitting the current classification were found and should be analysed in detail in future studies.

ARTICLE HISTORY
Received 8 October 2021
Accepted 1 August 2022

KEYWORDS
Calenduleae; chromosome numbers; Compositae; hybridization; nuclear DNA content; 2C; 1Cx

Introduction

The genus *Calendula* L. (Asteraceae, Calenduleae) is native to the Mediterranean basin, occurring from Atlantic Macaronesia to the Middle East (Norlindh 1946, 1977; Heyn et al. 1974; Nordenstam 1994; Nordenstam and Källersjö 2009). It comprises about 14 species (approx. 31 taxa when subspecies are included), and is considered as one of the most complex and taxonomically difficult genera within the Asteraceae family to classify (Norlindh 1977; Heyn and Joel 1983; Nordenstam and Källersjö 2009, Nora et al. 2013, Gonçalves et al. 2018). Calendula plants bear sessile, alternate leaves, and produce solitary capitula, with internal yellow, orange, brown or violet-purple tubular flowers, functionally male, and external yellow or orange ligulate female flowers. The achenes are heteromorphic, with no pappus (Nordenstam 1994). Cytogenetically, the genus is highly variable, with chromosome numbers ranging dramatically from $2n = 14$ to ± 88 (Nora et al. 2013), and with several base numbers being assumed (namely $x = 7$, 8, 9, 11 and 15?; Darlington and Wylie 1955; Norlindh 1977; Heyn and Joel 1983; Nordenstam 1994). Traditionally, *Calendula* is divided into annual and perennial herbs. The annual herbs, *sensu* Heyn et al. (1974), include five species: *C. stellata* Cav. ($2n = 14$ chromosomes), *C. tripterocarpa* Rupr. ($2n = 30$), *C. arvensis* L. ($2n = 44$), *C. palaestina* Boiss. ($2n$ = ±88), and *C. pachysperma* Zoh ($2n$ = ±88). In contrast, the perennial taxa were divided into two groups (i) the *C. maroccana* group, and (ii) *C. incana* and *C. suffruticosa* (Ohle 1974, 1975a, 1975b). The *C. maroccana* group comprises four species (*C. eckerleinii* Ohle, *C. maroccana* Ball, *C. meuselii* Ohle, and *C. lanzae* Maire), with $2n = 18$ chromosomes (Ohle 1975a). The *C. incana* and *C. suffruticosa* groups are composed by 7 and 11 taxa, respectively, all with $2n = 32$ chromosomes (Ohle 1974, 1975b). However, it is accepted that *C. incana* and *C. suffruticosa* groups are artificial and very difficult to distinguish (Nora et al. 2013), henceforth we followed the nomenclature proposed by Meikle et al. (1976), Silveira et al. (2013), and Gonçalves et al. (2018), and included all the *C. incana* taxa under *C. suffruticosa*.

The taxonomic complexity results from high morphological variability in some of its taxa which are under active evolution. This variability results from high levels of hybridization,

CONTACT Paulo Silveira psilveira@ua.pt Departamento de Biologia e CESAM, Universidade de Aveiro, 3810–193, Aveiro, Portugal.

Published online 19 Jan 2023

frequently leading to occurrences of intermediate forms (Lanza 1919; Heyn and Joel 1983) and occurrences of different achene morphologies within the same taxon (e.g. *C. arvensis*), along with similar achene morphologies in different taxa (e.g. *C. arvensis* vs. *C. stellata*). The production of more than one type of fruit per plant, known as heterocarpy (Zohary 1950), and its intricate heredity, introduces further difficulties in the process of taxonomical classification of the genus. Hybridization and polyploidization events were proposed as the main mechanisms giving rise to new entities (Heyn et al. 1974; Nora et al. 2013). Hence, a wide range of intermediate forms arise from allopolyploidization, genome duplication, or dysploidy (Nora et al. 2013), and most taxa are recognised by several names under different taxonomic categories (Nègre 1958; Heyn and Joel 1983).

Since the publications of Heyn et al. (1974) on annual taxa of *Calendula*, and of Ohle (1974, 1975a, 1975b) on perennial taxa, few studies have followed. Recently, Nora et al. (2013) analysed the chromosome number and genome size of 11 *Calendula* taxa. A gradient in genome size between *C. incana* and *C. suffruticosa* groups, along with considerations regarding morphological relationships, led these authors to aggregate both species. Nora and co-authors (2013) also discussed the main mechanisms of evolution, considering that, in agreement with previous authors (Norlindh 1977; Heyn and Joel 1983), *C. maroccana* ($2n = 18$) and *C. stellata* ($2n = 14$) played a central role in the origin of several taxa. These species also belong to the main centre of diversity and evolution of the genus. Although Nora et al. (2013) provided information on the taxonomy and evolution of *Calendula*, the study involved a limited number of taxa. Later, Plume et al. (2015) used chloroplast markers (*atpI-atpH*) and the nuclear ribosomal internal transcribed spacer region (ITS), together with palynological data, to assess hybridization events between *C. maritima* and *C. suffruticosa* subsp. *fulgida*. This study, of a limited sample set, suggested that the hybrids are capable to back-cross with their parents and that hybridization may be placing *C. maritima* at risk of extinction *via* introgression (Plume et al. 2015). Plume (2015) also studied the phylogenetic relationships within *Calendula* using molecular markers, providing support for multiple origins of most polyploid taxa, a division of the genus into annual and perennial polyploid complexes, and a single origin of *C. officinalis*. Although these studies strongly support the idea that hybridization and polyploidization have been important in the speciation of *Calendula*, the relationships within each taxon are still unclear. More recently, Gonçalves et al. (2018) revised the genus for the Iberian Peninsula, recognising four species for this region: *C. arvensis*, *C. officinalis*, *C. tripterocarpa* and *C. suffruticosa*, for which they recognized nine subspecies, including two new ones (*C. suffruticosa* subsp. *trialata* and *C. suffruticosa* subsp. *vejerensis*). While both *C. arvensis* and *C. suffruticosa* are complex and variable species, Gonçalves et al. (2018) only recognized subspecies in the latter taxa, where clear patterns of variation, correlated with different geographical distributions and habitats, can be observed. In the case of *C. arvensis*, such patterns could not be observed, and, in many cases, populations with mixed morphologies have been found. This is in agreement with previous treatments by Heyn et al. (1974) for *C. arvensis*, and Ohle (1974, 1975a, 1975b), Meikle et al. (1976), and Silveira et al. (2013) for *C. suffruticosa*.

This study was conducted to extend the understanding of the relationships between *Calendula* taxa by sampling largely unexplored areas of the SW Mediterranean basin, which are important centres of diversification of the genus, especially in Morocco, but including also taxa from Algeria, Tunisia, Sicily and Israel. The following objectives were formulated: (a) to estimate the nuclear DNA content of the *Calendula* spp. from Morrocco, including also taxa from Algeria, Tunisia, Sicily and Israel, most of them for the first time; (b) to check how does genome size relate with chromosome number, ploidy levels and life cycle, and (c) to use genome size as an aid in the classification of the *Calendula* spp.

Material and methods

Plant material

An intensive field survey was conducted between 2009 and 2014, focusing on Morocco and the most important/accessible taxa from Algeria and Tunisia. Collection sites were selected from the literature, and by inspecting the labels of ca. 5 000 herbarium specimens (for full information on herbaria consulted, please check Gonçalves et al. 2018). In total, achenes and/or fresh leaves from 58 populations were collected in the field, supplemented with 19 *ex situ* germinated seedlings from several countries like Italy, Israel-Palestine, Lebanon, Morocco and Turkey (see Table 1), totalling 77 populations. At each site, we aimed to collect leaves from five individual plants; however, occasionally smaller number of individuals were analysed due to the small number of individuals available *in situ*. Taxa were identified according to Heyn et al. (1974) for annuals and Ohle (1975a, 1975b) for perennials, except that *C. incana* and its subspecies were included in *C. suffruticosa* (Meikle et al. 1976; Silveira et al. 2013). Some populations previously included by other authors under known taxa which showed distinct morphologies were treated in this work as distinct entities (e.g. *Calendula* sp.1, *Calendula* sp.2 and *Calendula* sp.3) whose description and typification will be published elsewhere. Vouchers were prepared and deposited in AVE, BM, RAB, MA, MARK, MPU, and AL.

Genome size assessments

Fresh leaves were collected in the field and stored at 4 °C until processed. A nuclear suspension was obtained for each sample by chopping approximately 100 mg of *Calendula* spp. and 50 mg of *Pisum sativum* "Ctirad" (internal reference standard), using a razor blade in a Petri dish containing 1 mL of ice-cold WPB buffer [200 mM Tris.HCl, 4 mM $MgCl_2.H_2O$, 2 mM EDTA $Na_2O.2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1% PVP–10 and 1% (v/v) Triton X–100, pH adjusted to 7.5, and stored at 4 °C, following Loureiro et al. (2007)]. The nuclear suspension was filtered through a 50 µm nylon mesh and 50 µg mL^{-1} of propidium iodide (Fluka, Buchs, Switzerland)

Table 1. *Calendula* specimens used in the flow cytometry analysis.

Taxa	Locality	Coordinates	Voucher No.
C. arvensis	*TUNISIA: Sfax, the city of Möez, between Sfax and Thaenae; May 2009, 10 m, CA16164 in *Silveira, P*.	34°40′57"N, 10°42′06"E	3072
	*MOROCCO: NCRPIS (USDA), PI578099 in *Silveira, P.*		3093
	*FRANCE: NCRPIS (USDA), PI597585 in *Silveira, P.*		3098
	*ITALY: Apulia, Valenzano, NCRPIS (USDA) PI597587 in *Silveira, P.*	41°02′ N, 16°53′ E	3100
	*GREECE: Oraiokastron, 13 km NW of Thessaloniki; NCRPIS (USDA) PI603109 in *Silveira, P.*	40°44′ N, 22°55′ E	3102
	*MALTA: Malta; March 2010, *Mifsud, S*. in *Silveira, P.*	35°53′ N, 14°26′ E	3116
	MOROCCO: Agadir, road N8 between Agalgal and Argana (road Marrakesh - Agadir); April 2011, 1037 m, *Silveira, P.*	30°56′ N, 9°04′ W	3129
	MOROCCO: Agadir, road N1 near Arhoud, between Agadir and Cap Rhir; April 2011, 11 m, *Silveira, P.*	30°36′53" N, 9°48′04" W	3132
	MOROCCO: Marrakech, near to the Airport; March 2013, 476 m, *Silveira, P. Gonçalves, ACRS*; *Ouhammou, A.*	31°35′00" N, 8°00′44" W	3284
	MOROCCO: Taroudant, 17 km W of Taroudant; March 2013, 176 m, *Silveira, P. Gonçalves, ACRS; Ouhammou, A.*	30°28′32" N, 9°02′05" W	3290
	*TURKEY: NCRPIS (USDA) PI578097 in *Plume, O.*		OP399
	*LEBANON: *Plume, O.*		OP448
	*LEBANON: *Plume, O.*		OP450
	*ITALY: Modica; *Plume, O.*		OP450
	*ITALY: Sicily; *Plume, O.*		OP463
	*MOROCCO: *Plume, O.*		OP470
	*LEBANON: *Plume, O.*		OP477
C. eckerleinii	MOROCCO: Ifrane, limestone outcrop 3 km N of Ifrane on the N8 road to Fez; April 2010, 1621 m, Silveira, P.	33°33′26" N, 5°05′51" W	3064
	MOROCCO: Meknes, Ain Leuh, road P7311, near l'Oued Er-Rbia; May 2014, 1505 m, Silveira, P. Gonçalves, ACRS	33°16′48" N, 5°20′23" W	3330
	MOROCCO: Timahdite, Foung Kheneg; May 2014, 1920 m, Silveira, P. Gonçalves, ACRS	33°09′10" N, 5°03′43" W	3331
	MOROCCO: Boulemane, 31 km from Ifrane, 48 km from Sefrou, 25 km of Boulemane, at the junction of Ifrane road; May 2014, 1572 m, Silveira, P. Gonçalves, ACRS	33°27′18" N, 4°51′25" W	3332
	MOROCCO: Fes, Jebel Zalagh; May 2014, 895 m, Silveira, P. Gonçalves, ACRS	34°06′19" N, 4°58′11" W	3333
C. lanzae	MOROCCO: Taroudant, S of Ait-Yazza; March 2013, 257 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°28′35" N, 8°48′03" W	3292
	MOROCCO: Taroudant, gravel on the margins of the river Tiout; March 2013, 424 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°23′48" N, 8°42′17" W	3293
C. maroccana	*MOROCCO: NCRPIS (USDA), PI607416 in Silveira, P.		3105
subsp. *maroccana*	MOROCCO: Asni, High Atlas, between Tizi-n-Test and Asni; April 2011, 1836 m, Silveira, P.	30°51′08" N, 8°21′56" W	3142
	MOROCCO: Asni, High Atlas, near Tazalt, between Asni and Tizi-n-Test; April 2011, 1250 m, Silveira, P.	30°58′52" N, 8°13′33" W	3143
	MOROCCO: Taroudant, between Tiout and Igherm; March 2013, 1365 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°12′34" N, 8°28′51" W	3294
	MOROCCO: Taroudant, between Tiout and Igherm, at 3 km from Igherm; March 2013, 1656 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°06′41" N, 8°27′50" W	3295
	MOROCCO: Taroudant, leaving Igherm to Taliouine; March 2013, 1569 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°41′16" N, 7°16′18" W	3298
	MOROCCO: Taroudant; March 2013, 1909 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°47′48" N, 7°31′38" W	3299
	MOROCCO: Tizi-n-Tichka, on the N9 almost at Tizi-n-Tichka, coming from Tachokchte; March 2013, 1351 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	31°24′53" N, 7°23′43" W	3300
C. maroccana subsp. *murbeckii*	MOROCCO: Marrakech, near to the house of the gazelle reserve; March 2013, 624 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	31°52′09" N, 7°57′08" W	3280
	MOROCCO: Marrakech, south of Barrage Lalla Takerhust; March 2013, 729 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	31°21′36" N, 8°09′46" W	3287
C. meuselii	MOROCCO: Meknes, between Moulay Idriss and n'zala des Beni-Ammar; April 2010, 852 m, Silveira, P. & Gonçalves, ACRS	34°05′09" N, 5°25′58" W	3063
C. pachysperma	*ISRAEL: Judea and Samaria Area, Samaria mountains; 871 m, IPGN 20 562 in Silveira, P.	32°12′12′′ N, 35°16′24 E	3121
C. palaestina	*ISRAEL: Mount Carmel, 472 m, IPGN 21 124 in Silveira, P.	32°44′33′′ N, 35°02′54′ E	3120
Calendula sp1	MOROCCO: Al Hoceima, Bokkoya; May 2014, 740 m, Silveira, P. Gonçalves, ACRS	34°54′46" N, 3°47′59" W	3339
Calendula sp2	MOROCCO: Taza; June 2012, 500 m, Silveira, P. Gonçalves, ACRS	34°07′50"N, 4°18′09" W	3263
Calendula sp3	MOROCCO: between Agadir and Cap Rhir; April 2011, 27 m, Silveira, P.	30°36′N, 9°46′W	3130
	MOROCCO: Agadir; April 2011, 12 m, Silveira, P.	30°37′34" N, 9°51′27"W	3134
	MOROCCO: Sidi Ifni; April 2011, 256 m, Silveira, P.	29°36′50"N,10°01′25"W	3137
	MOROCCO: Sidi Ifni; April 2011, 256 m, Silveira, P.	29°36′50"N,10°01′25"W	3138
C. stellata	TUNISIA: between Bir Bou Rekba and Hammamet; April 2009, 0 m, Silveira, P.	36°26′ N, 10°35′ E	3039a
	*MOROCCO: El Jadida; *Plume, O*.		OP319
	MOROCCO: Meknes, vicinities of Moulay Idriss Zerhoun; April 2010, 429 m, Silveira, P.	34°03′54" N, 5°30′19" W	3061
	MOROCCO: Meknes, vicinities of Moulay Idriss Zerhoun; April 2010, 429 m, Silveira, P.	34°03′54" N, 5°30′19" W	3062
	MOROCCO: Agadir, road Agadir - Marrakech, before the Abdelmoumen water dam; April 2010, 644 m, Silveira, P.	30°35′21" N, 9°20′53" W	3068
	MOROCCO: Marrakech, near to the house of the gazelle reserve; March 2013, 624 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	31°52′09" N, 7°57′08" W	3281
	MOROCCO: Khemisset, Oued Beht, 18 km of Khemisset, near Oued Beht bridge; May 2014, 132 m, Silveira, P. Gonçalves, ACRS	33°52′47" N, 5°55′49" W	3329
	MOROCCO: Zaio, road N2, between Zaio and Nador; May 2014, 213 m, Silveira, P. Gonçalves, ACRS	34°59′52" N, 2°49′47" W	3337
C. suffruticosa	*ITALY: Sicily, Ronciglio (Trapani); NCRPIS (USDA), PI597596 in Silveira, P.	38°01′ N, 12°29′ E	3101

(*Continued*)

Table 1. Continued.

Taxa	Locality	Coordinates	Voucher No.
	*ITALY: Sicily, Mount Erice near Trapani; NCRPIS (USDA), PI613021 in Silveira, P.	38°01′ N, 12°29′ E	3109
	MOROCCO: Tangier-Tetouan; June 2012, 1070 m, Silveira, P. Gonçalves, ACRS	35°28′50" N, 5°22′06" W	3261
	MOROCCO: Tangier-Tetouan; June 2012, 468 m, Silveira, P. Gonçalves, ACRS	35°35′26" N, 5°22′45" W	3262
	MOROCCO: Nador; June 2012, 562 m, Silveira, P. Gonçalves, ACRS	35°14′11" N, 2°58′29" W	3265
	MOROCCO: Tangier-Tetouan; June 2012, 90 m, Silveira, P. Gonçalves, ACRS	35°12′39" N, 4°39′46" W	3266
	MOROCCO: Tangier-Tetouan; June 2012, 10 m, Silveira, P. Gonçalves, ACRS	35°54′27" N, 5°28′54" W	3267
	MOROCCO: Tangier-Tetouan; June 2012, 3 m, Silveira, P. Gonçalves, ACRS	35°54′ N, 5°27′ W	3268
	SPAIN: Ceuta, Benzu: June 2012, 5 m, Silveira, P. Gonçalves, ACRS	35°54′06" N, 5°20′44" W	3269
	ALGERIA: Algiers, Plage de La Madrague; June 2013, 13 m, Silveira, P. Gonçalves, ACRS; Amirouche, R.	36°47′25" N, 2°53′56" E	3316
	ALGERIA: Algiers, near to the beach; June 2013, 13 m, Silveira, P. Gonçalves, ACRS; Amirouche, R.	36°43′42" N, 2°50′27" E	3317
	ALGERIA: Algiers; June 2013, 13 m, Silveira, P. Gonçalves, ACRS; Amirouche, R.	36°41′25" N, 2°47′37" E	3318
	ALGERIA: Tipasa; June 2013, 30 m, Silveira, P. Gonçalves, ACRS; Amirouche, R.	36°37′19" N, 2°24′27" E	3319
	ALGERIA: Gorge des Palestro; June 2013, 340 m, Silveira, P. Gonçalves, ACRS; Amirouche, R.	36°36′ N, 3°35′ E	3320
	ALGERIA: Djurdjura; June 2013, 1798 m, Silveira, P. Gonçalves, ACRS; Amirouche, R.	36°27′46" N, 4°00′00" E	3321
	MOROCCO: Taza, Taza, Ras-El-Ma, Sidi Msbar; May 2014, 1460 m, Silveira, P. Gonçalves, ACRS	34°07′58" N, 4°07′58" W	3334
	MOROCCO: Berkane, Beni Snassen, road between Zegzel - Tazarhine - Takerkoust; May 2014, 54 m, Silveira, P. Gonçalves, ACRS	34°50′01" N, 2°22′17" W	3335
	MOROCCO: Berkane, Beni Snassen, near Oued Zegzel; May 2014, 260 m, Silveira, P. Gonçalves, ACRS	34°52′40" N, 2°21′20" W	3336
	MOROCCO: Al Hoceima, Bokkoyas, Taoussarte; May 2014, 133 m, Silveira, P. Gonçalves, ACRS	35°13′08" N, 4°05′14" W	3340
	MOROCCO: Tangier-Tetouan, Jebel Kelti, Arifane; May 2014, 943 m, Silveira, P. Gonçalves, ACRS	35°17′04" N, 5°18′00" W	3341
C. tripterocarpa	MOROCCO: Agadir, near Abdelmoumen water dam, NE of Agadir; April 2010, Silveira, P.	30°39′48" N, 9°13′56" W	3065
	MOROCCO: Guelmim, on the road to Plage Blanche; April 2010, 170 m, Silveira, P.	28°58′36" N, 10°15′25" W	3066
	MOROCCO: Agadir, between Marrakesh and Agadir, road N8 after Chichaoua; April 2011, 552 m, Silveira, P.	31°22′ N, 8°49′ W	3128
	MOROCCO: Marrakech, near to the house of the gazelle reserve; March 2013, 624 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	31°52′09" N, 7°57′08" W	3282
	MOROCCO: Taroudant, 17 km W of Taroudant; March 2013, 176 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°28′32" N, 9°02′05" W	3289
	MOROCCO: Taroudant, between Tiout and Igherm, at 3 km from Igherm; March 2013, 1656 m, Silveira, P. Gonçalves, ACRS; Ouhammou, A.	30°06′41" N, 8°27′50" W	3296

*Accessions resulting from cultivation. Voucher specimens collected by the team were deposited at AVE, while those collected by Olofron Plume can be found at BH.

and 50 µg mL^{-1} of RNAse (Fluka, Buchs, Switzerland) were added to stain DNA and prevent staining of double-stranded RNA, respectively. The samples were incubated for 5-20 min. on ice before being analysed on a Beckman-Coulter EPICS XL flow cytometer (Beckman-Coulter, Hialeah, FL, USA) operating at 488 nm air-cooled argon-ion laser at 25 mW power; one run per preparation was performed, in which 5 000 particles were measured/recorded. 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. FL vs. fluorescence pulse height was used to eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets, while in FL vs. SS (log) a polygonal region was defined to include only intact nuclei. These regions were used to get all the other graphics. As a quality standard, only histograms with a coefficient of variation lower than 5% for G_1 peaks of both the sample and the standard species were considered. Whenever possible, five independent individuals per population were analysed; however, in some cases only two or three samples were analysed (due to their not being in good condition or the populations were very small). The holoploid genome size in mass values (2C in pg; *sensu* Greilhuber et al. 2005) was obtained by the ratio between G_1 mean peaks of *Calendula* spp. and *P. sativum*, multiplied by the genome size of the reference standard (2C = 9.09 pg; Doležel et al. 1998). The holoploid genome size in Mbp was also calculated using the conversion rate, 1 pg = 980 Mbp (Doležel et al. 2003). The monoploid genome size (1Cx; *sensu* Greilhuber et al. 2005) of each sample was calculated by dividing the 2C-value by the ploidy level of the corresponding sample (Greilhuber et al. 2005). Because genome sizes were obtained for several individuals which were also characterised karyologically (see below), DNA ploidy levels could be inferred for all individuals analysed, except for *C. tripterocarpa*.

Chromosome counts

Chromosome counts were used to confirm the ploidy levels given based on genome size estimates, following the squash method described in Nora et al. (2013), with some modifications. Briefly, seeds were germinated on wet filter paper in Petri dishes at room temperature (20–25 °C). One week later, seedlings were potted in Jiffy-7 pots (www.jiffy.com) and maintained in homogeneous conditions (20 ± 2 °C, with a light intensity of 60 ± 5 mol m^{-2} s^{-1}).

Young root tips were pre-treated with ice-cold water for 12 h, fixed in a cold mixture of absolute ethanol and glacial acetic acid (3:1, v/v) at room temperature for 24 h, and then kept in the same fixative at 4 °C. Chromosome spreads were obtained either by the squash method described in Nora et al. (2013) or by the squash technique according to Ribeiro et al. (2016). Slides were analyzed either using Nikon Eclipse 80i microscope (Nikon Instruments, NY, USA), and images of chromosome spreads were acquired with a Leica digital camera DC200 (Gmbtt Leica Microsystems GmbH, Wetzlar, Germany) and processed using the Leica IM1000 v.1.1 software (Leica Microsystems AG, Heerbrugg, Switzerland) or using the epifluorescence microscope Imager Z1 (Zeiss) with the DAPI appropriate filter. Images were captured by the monochromatic camera AxioCam Hrm (Zeiss) through the AxioVision software (Zeiss) and processed with the Image J software. For each accession/sample, at least three plants were used to assess chromosomes numbers.

Statistical analysis

Descriptive statistics were calculated for genome size data (mean, standard deviation of the mean (SD), coefficient of variation (CV = SD/Mean), and minimum and maximum values of the holoploid (2C, pg) and monoploid (1Cx) genome sizes, in pg and Mbp). Combined bar charts of genome size variations were analysed separately for chromosome number, ploidy level, life cycle, and *C. maroccana* vs. *C. suffruticosa* group.

Prior to the evaluation of statistical differences, normality and homoscedasticity of data was checked. Since normality was never meet, non-parametric tests were used: Kruskal-Wallis one-way Analysis of Variance on Ranks test, followed by Dunn's test for multiple comparisons. Statistical analyses were performed in Sigmaplot (Systat Software, Inc, San Jose, California, USA).

Results

Chromosome counts, ploidy level and life cycle

The chromosome numbers analysed in the present study are summarised in Table 3, while chromosome numbers published elsewhere are summarised in Table 2. Figure 1 shows metaphase cells with clear heterochromatic chromocenters for two of the species observed in this study.

Based on chromosome numbers and genome size analyses, we detected three ploidy levels in *Calendula* (Table 3), namely: diploidy, which was the most common ploidy level, and was present in *C. stellata* ($2n = 2x = 14$ chromosomes), *C. eckerleinii*, *C. maroccana* subsp. *maroccana*, *C. maroccana* subsp. *murbeckii*, *C. meuselii*, *C. lanzae*, *Calendula* sp.1, *Calendula* sp.2, *Calendula* sp.3 ($2n = 2x = 18$); tetraploidy in *C. suffruticosa* ($2n = 4x = 32$) and *C. arvensis* ($2n = 4x = 44$); and the octoploidy in *C. palaestina* and *C. pachysperma* ($2n = 8x = \pm 88$). *Calendula tripterocarpa* is known from the literature to present 30 chromosomes and is said to be diploid, but its monoploid genome size points towards triploidy, or tetraploidy, we are not sure, so, in this study, no inference about the ploidy level of this species was made. The most common chromosome number was $2n = 2x = 18$, which was present in eight of the fourteen taxa analysed (Table 3).

The studied *Calendula* taxa were divided according to their life cycle. While *C. stellata*, *C. lanzae*, *C. maroccana* subsp. *murbeckii, Calendula* sp.3, *C. tripterocarpa*, *C. arvensis*, *C. pachysperma* and *C. palaestina* usually present an annual life cycle, *C. eckerleinii*, *C. maroccana* subsp. *maroccana*, *C. meuselii*, *Calendula* sp.1, *Calendula* sp.2 and *C. suffruticosa* are mostly perennial (Table 3).

Inter- and intraspecific variation in holoploid genome size

A total of 328 accessions (each one from an individual plant from 77 populations belonging to 14 taxa) were analysed by flow cytometry (Table 1 and Table 3). Fluorescence histograms of genome size assessments yielded well-defined peaks for both *Calendula* samples and the internal reference standard (Figure 2). Coefficients of variation (CVs) of most (8) of the studied (14) taxa were lower than 5%.

The mean 2C-values varied 6-fold from 1.37 pg in diploid *C. maroccana* subsp. *maroccana* to 8.26 pg in octoploid *C. palaestina*, with an overall mean of 2.84 ± 1.32 pg (Table 3). Significant differences in 2C-values ($H_{13} = 307.312$; $p < 0.001$) were obtained when comparing all the taxa studied (Table 3).

Mean 2C-values were found to be significantly and positively correlated with chromosome numbers ($R^2 = 0.703$, $p < 0.001$), which ranged from 1.59 pg in *C. maroccana* subsp. *maroccana* with 2n = 18 chromosomes, and 2.11 pg in *C. stellata* with 2n = 14 chromosomes to 7.84 pg in *C. palaestina* with $2n = 88$ chromosomes. The analysis of variance also confirmed significant differences ($H_5 = 287.321$, $p < 0.001$) between holoploid genome sizes and different chromosome numbers (Table 3 and Figure 3a). Mean 2C-value increased with ploidy levels, ranging from 1.88 ± 0.25 pg in diploids, 3.63 ± 0.95 pg in tetraploids, to 7.41 ± 0.56 pg in octoploids. *C. tripterocarpa*, with 3.53 ± 0.95 pg, is considered here as having an unknown ploidy level and, therefore, was excluded from this analysis. Mean 2C-value revealed significant differences ($H_2 = 223.360$; $p < 0.001$) between different ploidy levels (Figure 3c). A significant correlation between 2C-value and different ploidy levels could also be detected ($R^2 = 0.642$, $p < 0.001$).

Perennial taxa presented significantly lower 2C-value (2.46 ± 0.73 pg) than the annual ones (3.30 ± 1.69 pg) ($H_1 = 26.305$; $p < 0.001$; Figure 4a). Therefore, a significant correlation ($R^2 = -0.488$, $p < 0.001$) between holoploid genome size and life cycle was detected.

Variation in holoploid genome size ($H_1 = 166.257$; $p < 0.001$) was observed between the *C. maroccana* and *C. suffruticosa* groups (Figure 4c). Mean 2C-value varied from 1.80 ± 0.23 pg in *C. maroccana* group to 3.09 ± 0.21 pg in *C. suffruticosa* group, differing by ~1.72-fold. In the *C. maroccana* group the 2C values ranged from 1.37 pg in *C. maroccana* subsp. *maroccana* to 2.33 pg in *C. maroccana* subsp. *murbeckii and Calendula*

Table 2. Chromosome numbers reported for *Calendula* taxa from the SW Mediterranean region.

Taxa	Country/region	Chromosome number report		References
		n	2*n*	
C. arvensis	Mediterranean basin	18[1]	14[3]	[1](Negodi 1937)
		22[2]	18[3]	[2](Meusel and Ohle 1966; Heyn and Joel 1983; Aparicio 1989)
			44[4]	[3](Humphries et al. 1978)
				[4](Meusel and Ohle 1966; Marchi et al. 1974; Diaz Lifante et al. 1992; Vogt and Oberprieler 1993, 2008, 2012)
C. eckerleinii	Morocco		18	(Ohle 1975a; Vogt and Oberprieler 2008)
C. lanzae	Morocco		18	(Ohle 1975a)
C. maroccana subsp. *maroccana*	Morocco		18[1]	[1](Ohle 1975a, 1975b; Oberprieler and Vogt 1993; Vogt and Oberprieler 2012; Nora et al. 2013)
			32[2]	[2](Valdés and Parra 1997)
C. maroccana subsp. murbeckii	Morocco		18[1]	[1](Meusel and Ohle 1966; Ohle 1975a)
			32[2]	[2](Fedorov 1969)
C. meuselii	Morocco		18	(Ohle 1975a)
C. pachysperma	Israel, Palestine	±43[1]	±85[2]	[1](Heyn and Joel 1983)
			±88[3]	[2](Heyn et al. 1974)
				[3](Pazy 2000)
C. palaestina	Israel, Palestine	±43[1]	±85[2]	[1](Heyn and Joel 1983)
				[2](Heyn et al. 1974)
C. stellata	Morocco, Algeria, Tunisia, Italy	7[1]	14[2]	[1](Negodi 1937; Meusel and Ohle 1966; Humphries et al. 1978; Talavera et al. 1984; Aparicio 1989; Ruiz de Clavijo 1990)
			44[3]	[2](Meusel and Ohle 1966; Heyn et al. 1974; Ohle 1975a; Humphries et al. 1978; Oberprieler and Vogt 1993; Vogt and Oberprieler 1993, 2008, 2012; Nora et al. 2013)
				[3](Meusel and Ohle 1966)
C. suffruticosa	Morocco, Algeria, Tunisia, Portugal, Spain	9[1]	32[4]	[1](Meusel and Ohle 1966)
		14[2]		[2](Negodi 1937)
		16[3]		[3](Meusel and Ohle 1966; Ohle 1974, 1975b; Aparicio 1989)
				[4](Talavera et al. 1984; Vogt and Oberprieler 2008)
C. tripterocarpa	Morocco, Algeria, Tunisia, Spain	7[1]	30[4]	[1](Negodi 1937)
		14[2]	30 + 2B[5]	[2]Reese (1957)
		15[3]	54[6]	[3](Meusel and Ohle 1966; Heyn et al. 1974; Heyn and Joel 1983; Aparicio 1989)
				[4](Meusel and Ohle 1966; Heyn et al. 1974; Diaz Lifante et al. 1992)
				[5](Oberprieler and Vogt 1993; Vogt and Oberprieler 2008)
				[6](Dalgaard 1986)

sp.3 (Table 3). Significant differences (H_7 = 90.641; $p < 0.001$) within *C. maroccana* group were obtained. In the *C. suffruticosa* group 2C-values varied from 2.71 to 3.62 pg (Table 3).

Inter- and intraspecific variation in monoploid genome size

Significant differences in 1Cx-values (H_{12} = 251,338; $p < 0.001$) were obtained when comparing all the taxa studied (Table 3). The mean 1Cx-values varied 1.69-fold from 0.77 pg in *C. suffruticosa* to 1.30 pg in *C. arvensis*, with an overall mean of 0.93 ± 0.18 pg (Table 3). *C. tripterocarpa* was excluded from these analyses.

Significant differences (H_4 = 199.818, $p < 0.001$) between 1Cx-value and different chromosome numbers were obtained (Figure 3b), corroborated by a generally negative significant correlation (R^2 = −0.621, $p < 0.001$) between 1Cx-value and chromosome number.

The mean 1Cx-values were similar among ploidy levels, although diploids presented values of 0.94 pg, slightly higher than tetraploids 0.91 pg and octoploids 0.93 pg (Figure 3d). However, 1Cx-values of diploids varied 1.72-fold between 0.68 pg in *C. maroccana* subsp. *maroccana* to 1.17 pg in *C. maroccana subsp. murbeckii*; in tetraploids, 1Cx-values varied 2.07-fold between 0.68 pg in *C. suffruticosa* to 1.41 pg in *C. arvensis*; and in the octoploids 1Cx-values varied 1.18-fold between 0.87 pg in *C. pachysperma* to 1.03 pg in *C. palaestina* (both with 2*n* = ±88). Significant differences in 1Cx-values were found between the different ploidy levels (H_2 = 13.657, $p < 0.001$; Figure 3d), although no statistically significant differences at $\alpha > 0.05$ where detected according to Dunn's multiple comparison test. Additionally, a significant negative correlation between ploidy and 1Cx values (R^2 = −0.538, $p < 0.001$) was found.

Annuals presented significantly (H_1 = 218.835, $p < 0.001$) higher (1.10 ± 0.14 pg) 1Cx-values than perennials (0.80 ± 0.07 pg) (Figure 4b), although no significant correlation (R^2 = −0.061, $p = 0.662$) was obtained. The 1Cx-values of annuals varied 1.64-fold from 0.86 pg in *C. lanzae* to 1.41 pg in *C. arvensis*, while for perennials this value varied 1.43-fold from 0.68 pg in *C. suffruticosa* and C. *maroccana* subsp. *maroccana* to 0.97 pg in *C. eckerleinii*.

Interspecific variation in monoploid genome size (H_1 = 81.674; $P =< 0.001$) was observed between *C. maroccana* and *C. suffruticosa* groups (Figure 4d). Mean 1Cx-value differed by approximately 0.13-fold, from 0.77 pg in *C. suffruticosa* group to 0.90 pg in *C. maroccana* group. Intertaxa variation in 1Cx-value was also found in the *C. maroccana* group (H_7 = 90.641; $p < 0.001$).

Cytogeographic patterns of Calendula taxa

The most widespread taxon is the tetraploid *C. arvensis* (not represented in the map), which is ruderal. In some cases, it

Table 3. Genome size variation among the *Calendula* taxa studied (2C-values and 1Cx-values, both given in mass values (pg) and in Mbp).

Taxa	Life cycle	*nind.*	*npop.*	*2n*	Ploidy	2C-value					1Cx-value				
						2C (pg) ± S.D.	Min.	Max.	CV (%)	2C (Mbp)	1Cx (pg) ± S.D.	Min	Max	CV%	1Cx (Mbp)
C. arvensis L.	A	35	17	44	*4x*	5.20 ± 0.29^{a}	4.72	5.65	5.57	5086	1.30 ± 0.07^{a}	1.18	1.41	5.57	1272
C. eckerleinii Ohle	P	24	5	18	*2x*	1.74 ± 0.08^{bc}	1.62	1.94	4.78	1700	0.87 ± 0.04^{bc}	0.81	0.97	4.78	850
C. lanzae Maire	A	8	2	18	*2x*	1.85 ± 0.08^{bc}	1.72	1.99	4.34	1811	0.93 ± 0.04^{abc}	0.86	0.99	4.34	905
C. maroccana (Ball) B. D. Jacks. subsp. *maroccana*	P	37	8	18	*2x*	1.59 ± 0.14^{c}	1.37	1.83	8.80	1555	0.79 ± 0.07^{c}	0.68	0.91	8.80	777
C. maroccana subsp. *murbeckii* (Lanza) Ohle	A	16	2	18	*2x*	2.07 ± 0.14^{abc}	1.88	2.33	6.83	2024	1.03 ± 0.07^{ab}	0.94	1.17	6.83	1012
C. meuselii Ohle	P	9	1	18	*2x*	1.71 ± 0.03^{bc}	1.66	1.74	1.70	1670	0.85 ± 0.01^{bc}	0.83	0.87	1.70	835
C. pachysperma Zohari	A	3	1	88	*8x*	8.19 ± 0.09^{a}	8.09	8.26	1.09	8014	1.02 ± 0.01^{abc}	1.01	1.03	1.09	1002
C. palaestina Boiss.	A	6	1	88	*8x*	7.02 ± 0.03^{a}	7.00	7.08	0.49	6864	0.88 ± 0.004^{bc}	0.87	0.89	0.49	858
C. stellata Cav.	A	43	8	14*	*2x*	2.11 ± 0.10^{ab}	1.83	2.32	4.70	2066	1.06 ± 0.05^{ab}	0.91	1.16	4.70	1033
C. suffruticosa Vahl	P	101	20	32	*4x*	3.09 ± 0.21^{a}	2.71	3.62	6.65	3019	0.77 ± 0.05^{c}	0.68	0.91	6.65	755
C. tripterocarpa Rupr.	A	16	6	30	*?x*	3.53 ± 0.12^{a}	3.34	3.74	3.52	3449	–	–	–	–	–
Calendula sp.1	P	5	1	18*	*2x*	1.69 ± 0.11^{bc}	1.57	1.83	6.63	1652	0.84 ± 0.06^{bc}	0.79	0.92	6.63	826
Calendula sp.2	P	5	1	18*	*2x*	1.76 ± 0.06^{bc}	1.67	1.81	3.53	1724	0.88 ± 0.03^{bc}	0.83	0.91	3.53	862
Calendula sp.3	A	20	4	18*	*2x*	2.09 ± 0.16^{ab}	1.83	2.33	7.51	2045	1.05 ± 0.08^{ab}	0.91	1.16	7.51	1022

Information about life cycle, number of analysed individuals, number of analysed populations, chromosome number (2*n*) and ploidy levels, is also given for each taxon.
Life cycle (A = annual; P = perennial); Number of individuals (*nind.*); Number of populations (*npop.*); Chromosome number (2*n*; an * marks numbers observed in this study); ploidy level; mean holoploid genome size (2C, pg) and monoploid genome size (1Cx, pg), standard deviation (SD), minimum (min.) and maximum (max.), the coefficient variation (CV, %) and 2C and 1Cx-value in megabase pairs (Mbp). Different letters reveal statistically significant differences at $p < 0.05$ according to Dunn's multiple comparison test.

grows together with *C. tripterocarpa* and the diploid *C. stellata* in ruderal areas. The tetraploid *C. suffruticosa* was found essentially near the coastal range, extending from Morocco to Tunisia and southern Italy/Sicily (Figure 5), whereas some populations occurred inland at 1 000 to 2 000 m a.s.l. It is in Morocco that diploids harbour a greater taxonomic diversity. In addition, eight narrow endemics are also found in Morocco, namely *C. eckerleinii*, *C. meuselii,* and *Calendula* sp.2 (restricted to the Middle Atlas), the *C. maroccana* subsp. *maroccana* and *C. maroccana* subsp. *murbeckii* (in the High Atlas), *C. lanzae* and *Calendula* sp.3 (in the Anti-Atlas) and *Calendula* sp.1 (in the Rif Mountains) (Figure 5). The octoploid taxa *C. pachysperma* and *C. palaestina* seem restricted to Israel and Palestine.

Discussion

Our study provided novel insights into the cytogenetics of *Calendula* from Morocco and also include taxa from Algeria, Israel-Palestine, Italy, Lebanon, Tunisia and Turkey adding to the first contribution provided for the Iberian Peninsula by Nora et al. (2013).

Chromosome counts, ploidy level and life cycle

Most of the chromosome numbers published to date for *Calendula* are consistent within each taxon (Table 2). Those that are contradictory seem to invariably result from misidentifications due to the taxonomic complexity of the genus. For example, Humphries et al. (1978) reported $2n = 14$ and $2n = 18$ chromosomes for *C. arvensis* for Morocco, but the most frequent number is $2n = 44$ (Meusel and Ohle 1966; Oberprieler and Vogt 1993; Vogt and Oberprieler 2008, 2012). These contradictory counts resulted, most certainly, from confusion with *C. stellata* ($2n = 14$) and *C. maroccana* group ($2n = 18$), since these two taxa occur in Morocco. Valdés and Parra (1997) reported $2n = 32$ for *C. maroccana*, however, the material deposited in SEV (140 824) herbaria from "Beni Snassen" (Morocco) was later identified as *C. suffruticosa*, which possess $2n = 32$ chromosomes. This is in accordance with former reports by Vogt and Oberprieler (2008) of *C. suffruticosa* from "Beni Snassen" (Morocco). Meusel and Ohle (1966) reported $2n = 18$ for *C. suffruticosa* from High Atlas (Morocco), but later this taxon was included by the authors in the *C. maroccana* group. In the case of *C. tripterocarpa* $2n = 30$ (Meusel and Ohle 1966; Heyn et al. 1974; Diaz Lifante et al. 1992), $2n = 30 + 2B$ (Oberprieler and Vogt 1993) or $2n = 54$ (Dalgaard 1986) were reported. But, in this case, more research is required namely, to check if those accessions belong to the same taxonomical entity. The remaining chromosome numbers agree with previously published counts for the genus (see Table 2).

High cytogenetic diversity has been detected in this study. Three ploidy levels harbouring different basic chromosome numbers were detected among the 14 taxa studied (Table 3 and Table 2). Despite considered as diploid by previous authors (Heyn and Joel 1983; Nora et al. 2013), our results suggest that *C. tripterocarpa* is likely a tetraploid. However,

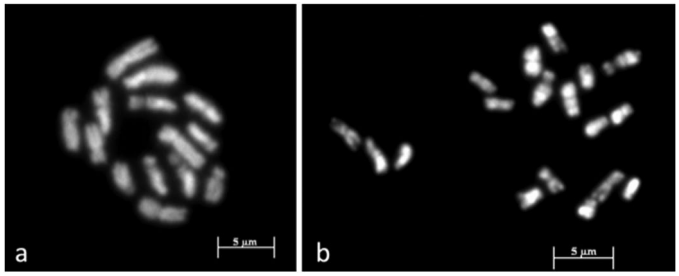

Figure 1. Representative somatic C-metaphase cells. (a) *C. stellata* with $2n = 14$ chromosomes (population 3061); (b) *Calendula* sp.1 with $2n = 18$ chromosomes (population 3339).

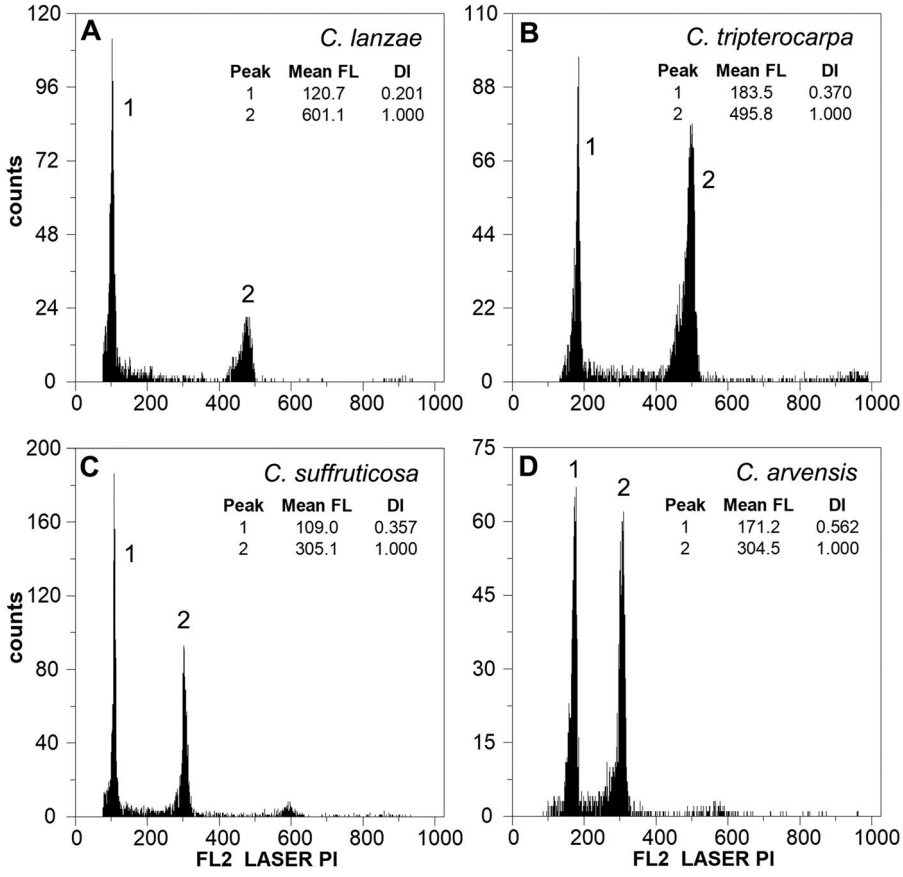

Figure 2. Representative flow cytometric histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from the internal reference standard and the *Calendula* spp.: A. *C. lanzae* (2n = 2x = 18), B. *C. tripterocarpa* (2n = 30), C. *C. suffruticosa* (2n = 4x = 32), D. *C. arvensis* (2n = 4x = 44). In these histograms the following peaks are marked: 1 – nuclei at G0/G1 phase of the sample; 2 – nuclei at G0/G1 phase of internal standard (*Pisum sativum* cv. Ctirad with 2C = 9.09 pg DNA). The mean channel number (mean FL), DNA index (DI = mean channel number of sample/mean channel number of reference standard) of each peak are also provided.

no inference about its ploidy was made, until further and more conclusive studies are performed, and this species is considered here to have an unknown ploidy level ($2n = x? = 30$). Previous studies have suggested that *C. tripterocarpa* may have originated after hybridisation between *C. stellata* and *C. maroccana* x *C. stellata* hybrid ($2n = 16$) or *C. maroccana* after losing two chromosomes (Heyn and Joel 1983). However, Nora et al. 2013 suggested that dysploidy could have been involved, with a resulting theoretical genome size closer to the estimations obtained for *C. tripterocarpa*. Additionally, previous studies also propose that *C. palaestina* and *C. pachysperma* may have originated either 1) through hybridization between *C. arvensis* and *C. tripterocarpa*, followed by whole genome duplication, or 2) through whole genome duplication of *C. arvensis* (Heyn and Joel 1983; Nora et al. 2013). Here, our chromosome counts and genome size estimates point into different directions. While genome sizes seem to support the first scenario (i.e. hybridization and genome duplication event), where theoretical genome size (8.88 pg) is closer from the values obtained for example in *C. pachysperma* (8.19 pg), the chromosome counts support the whole genome duplication of *C. arvensis* (with $2n = 4x = 44$) in the origin of both *C. palaestina* and *C. pachysperma* (both with $2n = 8x = 88$). After genome duplication event(s), the genome of the new octoploids would have suffered strong genome downsizing resulting in the current genome sizes observed for these two

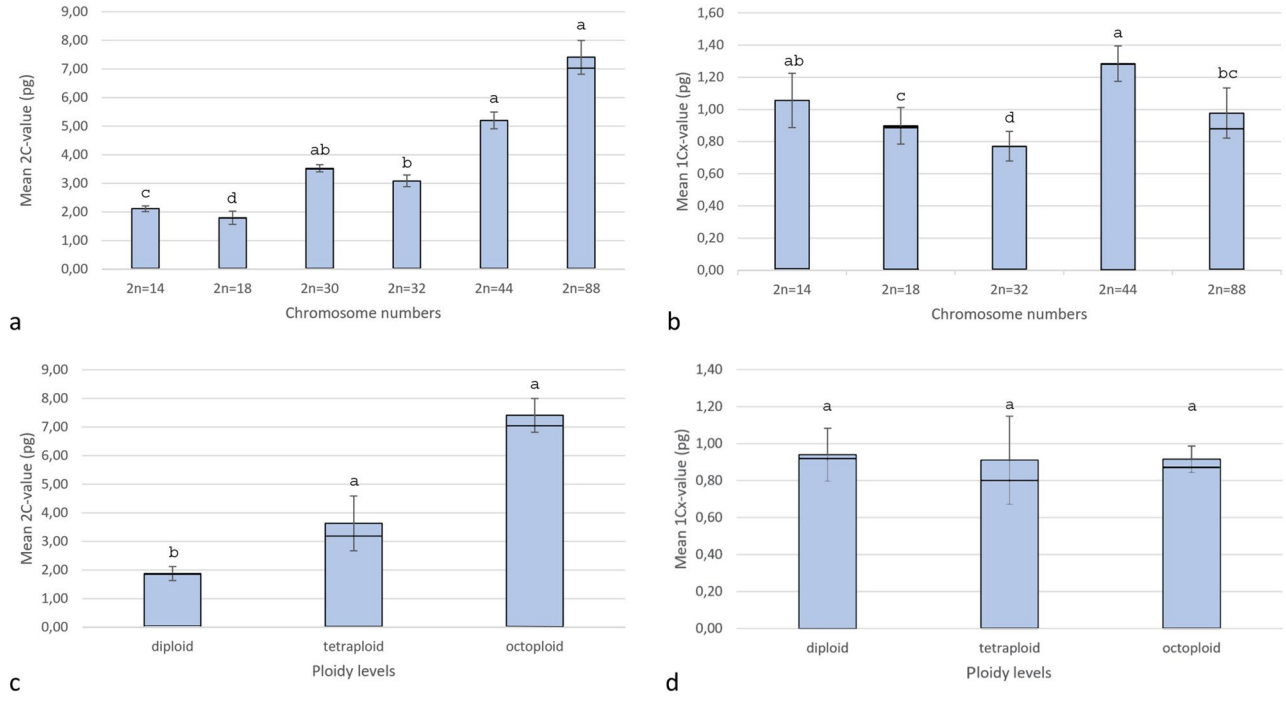

Figure 3. Bar charts showing genome size estimates. (a, c) holoploid (2C-values); (b, d) monoploid (1Cx-values) genome size variation in the *Calendula* taxa studied. (a, b) chromosome numbers $2n = 14$, 18, 30 (only in a), 32, 44 and ±88; (c, d) ploidy categories diploids, tetraploids and octoploids; Each bar represents an average value, combined with the error (± standard deviation). Different letters reveal statistically significant differences at $\alpha > 0.05$ according to Dunn's multiple comparison test.

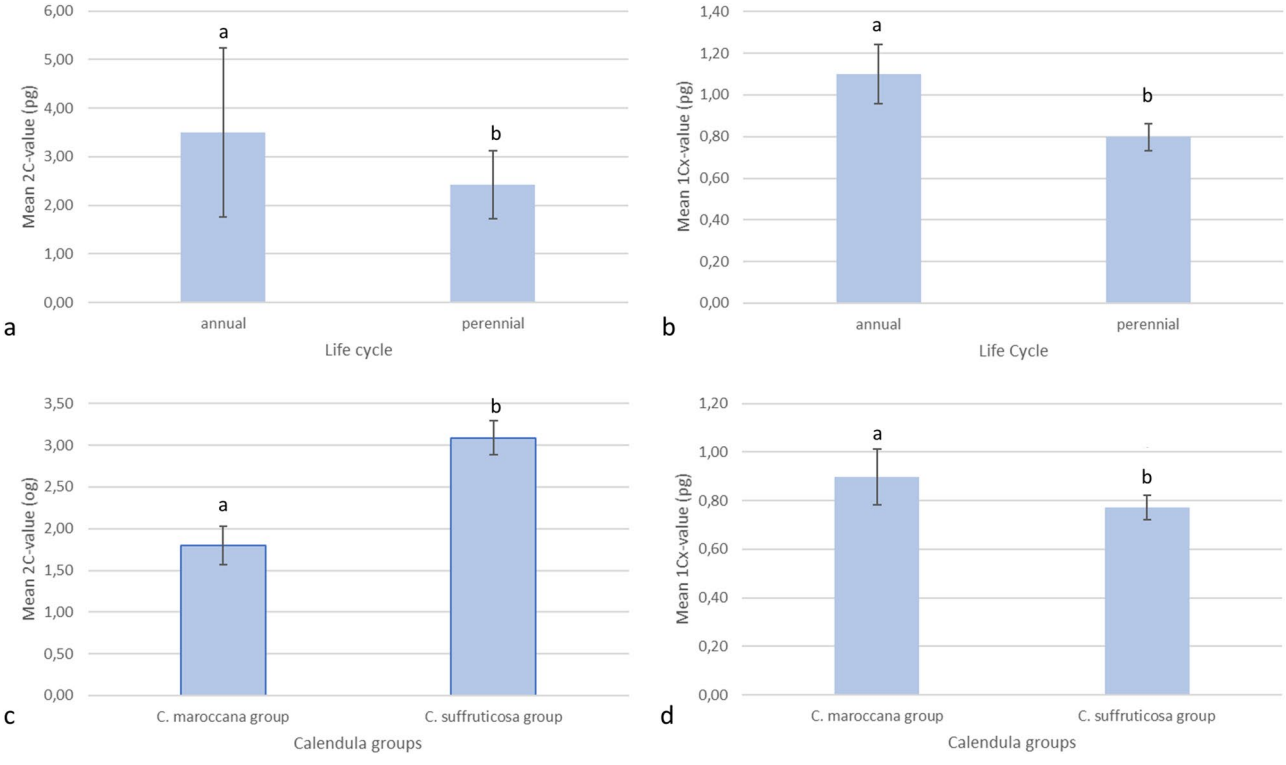

Figure 4. Bar charts showing holoploid (2C-values) and monoploid (1Cx-values) genome size variation in *Calendula* taxa. (a, b) life cycle: "annual" and "perennial"; (c, d) *C. maroccana* and *C. suffruticosa* groups representing different taxa and cytotypes of *Calendula*. Each bar represents an average value, combined with the error (± standard deviation). Different letters reveal statistically significant differences at $\alpha > 0.05$ according to Dunn's multiple comparison test.

octoploid species (Table 3). Additionally, the high morphological resemblance between the tetraploid *C. arvensis* and the octoploids *C. palaestina* and *C. pachysperma* may further support an autopolyploid origin for the two octoploid species. Indeed, the main characters differentiating *C. arvensis* and the octoploid species are the outer enlarged achenes

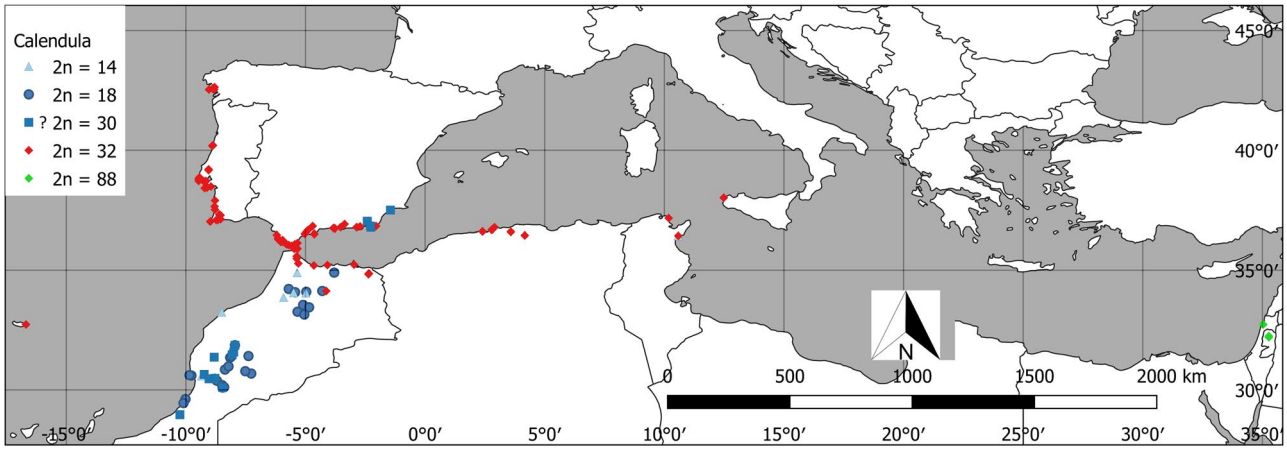

Figure 5. Geographical distribution of all studied *Calendula* populations, that could be georeferenced, in the study area (Morocco, Algeria, Tunisia, Italy and Israel-Palestine), including Iberian populations from Nora et al. (2013), except the widespread *C. arvensis* and the cultivated *C. officinalis*. Different symbols represent different chromosome numbers. Different colours represent ploidy level of each population: diploids = blue; tetraploids = red; octoploids = green.

(Heyn et al. 1974), which may be related with adaptation to more arid environments such as the ones where *C. palaestina* and *C. pachysperma* usually grow, and were also observed in other *Calendula* taxa from the south of Morocco and the Canary islands (P.Silveira person. obs.). Furthermore, we also agree with Heyn et al. (1974) that these octoploid species seem very close related, based on morphological and karyological characters, in a mode that resembles the affinity between the typical *C. arvensis* and its "alata" form), and also between *C. algeriensis* Boiss. & Reut. and *C. stellata*, which are considered a single species (Heyn et al. 1974).

Calendula is one of the smallest genera of Asteraceae, presenting only 14 species, usually divided into annual and perennial plants. However, this seems to be an entirely artificial arrangement, since these groups seem to include plants from distinct evolutionary lines, like the annual diploids like *C. stellata* ($2n = 14$) and *C. lanzae* ($2n = 18$); the tetraploid *C. arvensis* ($2n = 44$); the octoploids *C. pachysperma* and *C. palaestina* ($2n = \pm 88$), and *C. tripterocarpa* ($2n = 30$), with an unknown ploidy level. The same happens in the perennials, which include the *C. maroccana* diploid group of taxa (with $2n = 18$), and the tetraploid *C. suffruticosa* ($2n = 32$). This explanation, that some species belong to different evolutionary lines, was proposed because some of these plants, like *C tripterocarpa* and *C. stellata*, have higher genome sizes than expected, considering their number of chromosomes, when compared with other taxa (Nora et al. 2013).

Interestingly, our genome size estimates, coupled with chromosome counts, bring some insights also in the core group of diploid taxa. Clearly, we can recognize a group with $2n = 18$ chromosomes, the *C. maroccana* group, including both annual and perennial taxa such as *C. maroccana* subspecies, *C. eckerleinii*, *C. lanzae*, *C. meuselii* and several undescribed taxa. This group shares the same chromosome number and presents a high variation in genome size, with average sizes ranging from 1.59 pg in *C. maroccana* subsp. *maroccana* and 2.07 pg in *C. maroccana* subsp. *murbeckii* (and 2.09 pg in an undescribed taxon; Table 3). The variability of genome sizes found in *C. maroccana* group may, thus, support the hypothesis proposed by Heyn and Joel (1983) and discharged by Nora et al. (2013) in which *C. stellata* originated from *C. maroccana*. Contrarily to Heyn and Joel (1983) proposal where there were several chromosome losses, the genome size estimates support chromosome fusion (and maintenance of genome size).

The results obtained here clearly identify the need for detailed cytogenetic studies using *in situ* hybridization techniques (such as FISH and GISH) and molecular tools to reconstruct the evolutionary history and phylogenetic relationships between *Calendula* species.

Inter- and intraspecific variation in genome size

The estimates of genome size, including new assessments for *C. eckerleinii*, *C. lanzae*, *C. maroccana* subsp. *murbeckii, C. meuselii*, *C. pachysperma, C. palaestina*, and some subspecies of *C. suffruticosa* are presented. The results cover all the genome size variation present in the genus since we included genome size estimates - in this study and Nora et al. (2013) - of all the 11 currently recognised *Calendula* species plus three new entities yet undescribed. Variation in 2C-value among taxa was lower than that reported in previous studies of the genus (Garcia et al. 2013a; Nora et al. 2013). However, the estimates obtained for *C. arvensis*, *C. stellata*, *C. suffruticosa*, and *C. tripterocarpa* were consistent with most of the previous reports. The only exception is a record of $2C = 2.88$ pg for a $2n = 44$ accession of *C. arvensis* from France (BCN 88579) by Garcia et al. (2013b), which should be subjected to more research, and confirmation, since we have not one, but many accessions of *C. arvensis* whose estimations average 5.41 pg, and $2n = 44$ is the most frequent chromosome number recorded for this taxon (Meusel and Ohle 1966; Marchi et al. 1974; Diaz Lifante et al. 1992; Vogt and Oberprieler 1993, 2008, 2012).

Our results indicate that genome size could provide information taxonomically useful, namely, by helping in the process of placing species in the appropriate group (like *C. suffruticosa* or *C. maroccana* group) or by pinpointing new taxa, and also that can be used in evolutionary studies.

The monoploid genome size across the species suggest that species with the basic number 8 and 9 have 1Cx = 0.77 pg and 0.90 pg, respectively. Considering that species with the basic chromosome number $x = 9$ are predominant in *Calendula*, and present the most primitive morphological characters, especially the achenes (Norlindh 1946), we can suggest that all other basic chromosome numbers proposed for this genus are, probably, derived numbers. Considering the similarities in monoploid genome sizes and multiple basic chromosome numbers, dysploidy is present in the genus (Heyn and Joel 1983; Nora et al. 2013).

Our data confirm previous reports that both annuals and perennials encompass several chromosome numbers and ploidy levels. However, annuals are more variable cytogenetically than perennials, as they include diploids, tetraploids, and even two octoploid species. Correlations between genome size and life cycle have been widely discussed, although no general conclusions have emerged (Vallès et al. 2013). Plants with an annual life history are preferentially associated with inbreeding species characterised by low genome size than outbreeders (Bennett 1972; Garnatje et al. 2004; Grotkopp et al. 2004). However, annual plants from Asteraceae seem to show the opposite pattern (Vallès et al. 2013). In *Calendula*, a higher mean genome size was observed in annuals, agreeing with Torrell and Vallès (2001). However, most of the time such correlations between life cycle and nuclear DNA content do not exist (Torrell and Vallès 2001). While Plume (2015) found evidence to support the division of the genus into annual and perennial polyploid complexes, our data could not confirm this division, since plants with the same chromosome number may behave as annual or perennial (e.g. *C. maroccana* subsp. maroccana – perennial, *C. maroccana* subsp. *murbeckii* - annual), and both groups include plants with different chromosome numbers that do not seem to interbreed naturally (e.g. species of the *C. maroccana* group with any of the *C. suffruticosa* subspecies).

According to Ohle (1975a), *Calendula* is divided in two groups: I. *C. maroccana* and II. *C. suffruticosa* and *C. incana* (designated as *C. suffruticosa* in this work). On one hand, a higher 2C-value of the *C. suffruticosa* group was confirmed when compared with the *C. maroccana* group (Figure 4c), which is expectable given the differences in ploidy levels and chromosome numbers. Genome downsizing, i.e. a decrease in monoploid genome size was observed in the tetraploid *C. suffruticosa* (Figure 4d). The mechanism of evolution of *C. suffruticosa* is still poorly understood; however, it is hypothesised that its parental taxa originated an intermediate entity (through dysploidy from *C. maroccana* group) which then suffered a genome duplication event, originating an entity with $2n = 32$ chromosomes (Heyn et al. 1974; Nora et al. 2013). However, the monoploid genome size values might suggest that part of the *C. maroccana* group has evolved from perennial (with lower 1Cx-values) to annual taxa (having higher 1Cx-values). Interestingly, the pattern in monoploid genome size found within the genus is also found within the *C. maroccana* group, i.e. annual plants having larger monoploid genome sizes than perennials. This study also confirms that taxa from *C. maroccana* group played a central role in the origin of several taxa, through hybridization and polyploidization events (Nora et al. 2013).

Conclusion

The current study concludes the analysis of genome size variation in *Calendula* performed by the team encompassing all known species of the genus. The genome size of 13 species is provided, among which five were already known, eight are reported for the first time, and these include three putative new undescribed species. Chromosome numbers are reported for the first time for three, yet undescribed, new species, while previous reports for already known species were confirmed. Diploid, tetraploid and octoploid populations, most of them occupying the SW Mediterranean region, compose the genus. Holoploid genome sizes were found to be, at a certain extent, correlated with chromosome number and ploidy level. Evidences for genome downsizing with the increase in ploidy level were also detected. Knowledge of the geographical distribution and genome sizes were both important to understand the distribution patterns of *Calendula* taxa. However, further research, namely using molecular techniques, is needed to explore the evolutionary relationships between taxa and unravel their origins.

Acknowledgements

We thank anonymous reviewers for the suggestions, and William Smith for the English revision, that greatly improved the manuscript. Thanks are also due to several herbaria (ABH, AL (ENSA), ARAN, B, BC (IBB), BCN, BM, BONN, BR, C, COI, E, ELVE, FI, G, GAT, GDA (GDAC), HAL, JACA, JAEN, JE, K, LD, LEB, LISI, M, MA, MACB, MAF, MARK, MGC, MPU, O, P, PO, RAB, RNG, SALA, SANT, SEV, TFMC, US, VAL and W) for allowing the study or loaning plant material of *Calendula*. We are very grateful to several institutions that have authorized collections of *Calendula* specimens, namely: Instituto da Conservação da Natureza e das Florestas (Portugal), Parque Nacional de las Islas Atlanticas de Galicia y Parque Natural de la Sierra de las Nieves (Spain), Jbilet Reserve (Morocco). We also thank for the achene samples provided by: Real Jardín Botanico de Madrid (CSIC), USDA, IPGN, Olofron Plume (Cornell University, Ithaca, United States), Stephen Mifsud (Malta) and Miguel Sequeira (Univ. of Madeira, Portugal).

Disclosure statement

The authors declare that they have no conflict of interest.

Funding

This study was partly funded by the Portuguese Foundation for Science and Technology and UNESCO Fund for financing the work of ACG with the scholarship SFRH/BD/51464/2011 and in the form of institutional resources provided by the FCT/MCTES for the financial support to CESAM (UIDP/50017/2020 + UIDB/50017/2020 + LA/P/0094/2020), through national funds. This research was also supported by POPH/FSE funds by the Portuguese Foundation for Science and Technology (FCT) to the LEAF (Linking Landscape, Environment, Agriculture and Food research Unit – UID/AGR/04129/2013), and with a starting grant to SC (IF/01267/2013), and Portuguese Foundation for Science and Technology and European Social

Fund financed the work of HO with the scholarship SFRH/BPD/111736/2015. Additional support was provided by SYNTHESYS, grant GB-TAF-2900, to PS, allowing the study of BM and K's Calendula collections.

ORCID

Ana Carla Gonçalves http://orcid.org/0000-0002-8085-1336
Helena Oliveira http://orcid.org/0000-0002-4673-0696
João Loureiro http://orcid.org/0000-0002-9068-3954
Sílvia Castro http://orcid.org/0000-0002-7358-6685
Teresa Ribeiro http://orcid.org/0000-0003-2487-5293
Leonor Morais-Cecílio http://orcid.org/0000-0001-9313-2253
Conceição Santos http://orcid.org/0000-0003-4129-6381
Paulo Silveira http://orcid.org/0000-0002-9253-5381

References

Aparicio A. 1989. Números cromosomáticos de plantas occidentales: 487–507. An Jard Bot Madr. 45:483–494.
Bennett MD. 1972. Nuclear DNA content and minimum generation time in herbaceous plants. Proc R Soc Lond B Biol Sci. 181:109–135.
Dalgaard V. 1986. Chromosome studies in flowering plants from Macaronesia. An Jard Bot Madr. 43:83–111.
Darlington CD, Wylie AP. 1955. Chromosome atlas of flowering plants. New York: The Macmillan Company.
Diaz Lifante Z, Luque T, Santa Barbara C. 1992. Chromosome numbers of plants collected during Iter Mediterraneum. Bocconea. 3:229–250.
Doležel J, Bartos J, Voglmayr H, Greilhuber J. 2003. Nuclear DNA content and genome size of trout and human. Cytometry A. 51(2):127–128; author reply 129.
Doležel J, Greilhuber J, Lucretti S, Meister A, Lysák MA, Nardi L, Obermayer R. 1998. Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann Bot (Oxford). 82:17–26.
Fedorov AA. 1969. Chromosome numbers of flowering plants. Academy of Sciences of the USSR, Moscow, the Komarov V.L. Botanical Institute, Nauka, Leningrad.
Garcia S, Garnatje T, Gras A, Gálvez F, Anadon A, Canela MÁ, Hidalgo O, Mas de Xaxars G, Pellicer J, Siljak-Yakovlev S, et al. 2013a. GSAD: GSAD: a genome size database in the Asteraceae. Cytometry Part A. 79A:401–404. A genome size in the Asteraceae database. Release 2.0. http://www.etnobiofic.cat/gsad_v2/index.php.
Garcia S, Hidalgo O, Jakovljević I, Siljak-Yakovlev S, Vigo J, Garnatje T, Vallès J. 2013b. New data on genome size in 128 Asteraceae species and subspecies, with first assessments for 40 genera, 3 tribes and 2 subfamilies. Pl Biosyst. 147(4):1219–1227.
Greilhuber J, Doležel J, Lysák MA, Bennett MD. 2005. The origin, evolution and proposed stabilization of the terms "genome size" and "C-value" to describe nuclear DNA contents. Ann Bot. 95(1):255–260.
Garnatje T, Vallès J, Garcia S, Hidalgo O, Sanz M, Canela MA, Siljak-Yakovlev S. 2004. Genome size in *Echinops* L. and related genera Asteraceae, Cardueae: karyological, ecological and phylogenetic implications. Biol Cell. 96(2):117–124.
Gonçalves AC, Castro S, Paiva J, Santos C, Silveira P. 2018. Taxonomic revision of the genus *Calendula* (Asteraceae) in the Iberian Peninsula and the Balearic Islands. Phytotaxa. 352(1):1–91.
Grotkopp E, Rejmánek M, Sanderson MJ, Rost TL. 2004. Evolution of genome size in Pines (Pinus) and its life-history correlates: supertree analyses. Evolution. 58(8):1705–1729.
Heyn CC, Dagan O, Nachman B. 1974. The annual *Calendula* species: taxonomy and relationships. Israel J Bot. 23:1201–1969.
Heyn CC, Joel A. 1983. Reproductive relationships between annual species of *Calendula*. Pl Syst Evol. 143:311–329.
Humphries CJ, Murray BG, Bocquet G, Vasudevan K. 1978. Chromosome numbers of phanerogams from Morocco and Algeria. Bot Not. 131:391–406.
Lanza D. 1919. Monografia del genere *Calendula* L. Atti R Acad Sci, Palermo. 11:1–166.
Loureiro J, Rodriguez E, Doležel J, Santos C. 2007. Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Ann Bot. 100(4):875–888.
Marchi P, Capineri R, D'Amato G. 1974. Numeri Cromosomici per la Flora Italiana. Inf Bot Ital. 6:303–319.
Meikle RD, et al. 1976. *Calendula* L. In: Tutin TG, Heywood VH, Burges NA, editors. Flora Europaea Notulae Systematicae ad Floram Europaeam Spectantes. Cambridge: Cambridge University Press. p. 206–207.
Meusel H, Ohle H. 1966. Zur Taxonomie und Cytologie der Gattung *Calendula*. Österr Bot Z. 113:191–210.
Negodi G. 1937. Caratteri nucleari e morfologici in alcune Calenduleae. (Compositae-Tubuliflorae). Ann Bot (Oxford). 21:510–518.
Nègre R. 1958. A propos de quelques *Calendula* marocains. Bull Soc Sci Nat Phys Maroc. 38:1–7.
Nora SV, Castro S, Loureiro J, Gonçalves AC, Oliveira H, Castro M, Santos C, Silveira P. 2013. Flow cytometric and karyological analyses of *Calendula* species from Iberian Peninsula. Pl Syst Evol. 299:853–864.
Nordenstam B. 1994. Calenduleae. In: Bremer K, editor. Asteraceae: cladistics & classification. Portland: Timber Press. p. 365–376.
Nordenstam B, Källersjö M. 2009. Calenduleae. In: Funk VA, Susanna A, Stuessy TF, Bayer RJ, editors. Systematics, evolution, and biogeography of compositae. Vienna: IAPT. p. 527–538.
Norlindh T. 1946. Studies in the Calenduleae. II. Phytogeography and interrelation. Bot Not. 1946:471–506.
Norlindh T. 1977. Calenduleae - systematic review. In: Heywood VH, Harborne JB, Turner BL, editors. The biology and chemistry of the compositae. London (UK): Academic Press. p. 961–987.
Oberprieler C, Vogt R. 1993. Chromosome numbers of North African phanerogams. II. Willdenowia. 23:211–238.
Ohle H. 1974. Beiträge zur Taxonomie der Gattung *Calendula* L. II. Taxonomische Revision der südeuropäischen perennierenden Calendula-Sippen. Feddes Repert. 85:245–283.
Ohle H. 1975a. Beiträge zur Taxonomie und Evolution der Gattung *Calendula* L. III. Revision der marokkanischen perennierenden Sippen unter Berücksichtigung einiger marokkanischer Annueller. Feddes Repert. 86:1–17.
Ohle H. 1975b. Beiträge zur Taxonomie und Evolution der Gattung *Calendula* L. IV. Revision der algerisch-tunesischen perennierenden *Calendula*-Sippen unter Berücksichtigung einiger marokkanisch-algerischer Annueller und der marokkanischen und südeuropäischen perennierende. Feddes Repert. 86:525–541.
Pazy B. 2000. Flora Palaestina: chromosome numbers. Israel J Pl Sci. 48:7–32.
Plume O. 2015. Hybridization, genome duplication, and chemical diversification in the evolution of *Calendula* L. (Compositae) [Ph.D Thesis]. Ithaca (NY): Cornell University.
Plume O, Raimondo FM, Troia A. 2015. Hybridization and competition between the endangered sea marigold (*Calendula maritima*, Asteraceae) and a more common congener. Pl Biosyst. 149:68–77.
Reese G. 1957. Über die Polyploidiespektren in der nord-saharischen Wüstenflora. Flora. 144:598–634.
Ribeiro T, Barrela RM, Bergès H, Marques C, Loureiro J, Morais-Cecilio L, Paiva JAP. 2016. Advancing Eucalyptus genomics: cytogenomics reveals conservation of Eucalyptus genomes. Front Plant Sci. 7:510.
Ruiz de Clavijo E. 1990. Números cromosomáticos de plantas occidentales, 619–630. Anales Jard Bot Madrid. 47:431–437.
Silveira P, Gonçalves AC, Santos C, Paiva J. 2013. Two lectotypifications and a new combination in *Calendula* (Asteraceae) for Flora Iberica. Phytotaxa. 145:47–53.
Talavera S, Devesa J, Fernández-Galiano E. 1984. Notas cariosistemáticas sobre plantas Norteafricanas. I. Compositae. Candollea. 39:271–280.
Torrell M, Vallès J. 2001. Genome size in 21 *Artemisia* L. species (Asteraceae, Anthemideae): systematic, evolutionary, and ecological implications. Genome. 44(2):231–238.

Valdés B, Parra R. 1997. Numeros cromossomaticos de plantas de Marruecos, I. Lagascalia. 20:161–166.

Vallès J, Canela MÁ, Garcia S, Hidalgo O, Pellicer J, Sánchez-Jiménez I. 2013. Genome size variation and evolution in the family Asteraceae. Caryologia. 66:221–235.

Vogt R, Oberprieler C. 1993. Chromosome numbers of North African phanerogams. I. Fl Medit. 3:187–210.

Vogt R, Oberprieler C. 2008. Chromosome numbers of North African phanerogams. VIII. More Counts in Compositae. Willdenowia. 38:497–519.

Vogt R, Oberprieler C. 2012. Chromosome numbers of North African phanerogams. X. Plants collected during Iter Mediterraneum V of OPTIMA in Morocco. Ann Naturhist Mus Wien B. 11:193–221.

Zohary M. 1950. Evolutionary trends in the fruiting head of Compositae. Evolution. 4:103–109.