JSE Journal of Systematics and Evolution

doi: 10.1111/jse.13025

Research Article

A cytosystematic study of the *Dianthus virgineus* complex (Caryophyllaceae) in the Central Mediterranean

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Received 26 June 2023; Accepted 7 September 2023

Abstract European wild carnations (*Dianthus*) are represented by a high number of taxa organized in unresolved taxonomies. In particular, taxa belonging to the *Dianthus virgineus* L. complex in the Central Mediterranean have been delimited mainly with qualitative morphological data and still await quantitative investigations, which are vital to understand boundaries and relations among plant diversity groups. Here, we examine the phenotypic features of nuclear genome organization testing for species boundaries in this complex. We have studied the chromosome number, the total haploid length (THL), and the relative genome size (RGS) in 122 populations belonging to 25 out of 33 taxa of the complex. All the studied populations have $2n = 2x = 30$ chromosomes, and the THL ranges from 14.09 to 20.71 μm. Genome size estimations support the absence of polyploidization events, but show a certain degree of variation (0.318–0.423 arbitrary units). The RGS variation is not in agreement with current taxonomic treatment, but rather shows a geographical pattern, with higher values in Sicily and Sardinia. No correlation between the THL and the RGS was detected, possibly due to the stable chromosome number and the small size of chromosomes. A number of evolutionary unique groups lower than the number of currently accepted taxa may be hypothesized.

Key words: cytogenetics, genome size, karyosystematics, Mediterranean, plant evolution, taxonomy.

1 Introduction

The majority of wild carnation species (*Dianthus* L., Caryophyllaceae) belongs to a lineage rapidly diversified in Eurasia (Valente et al., 2010), with usually weak interspecific reproductive barriers (Carolin, 1957). Consequently, rapid and reticulated evolution hampered morphological species delimitations, leading to artificial classifications (Tutin, 1993) including many complexes of highly polymorphic, morphologically similar taxa (Bacchetta et al., 2010; Domina et al., 2017; Castro et al., 2022; Terlević et al., 2023). Fassou et al. (2022) pointed out that the high diversification rate of *Dianthus* may be linked to the differentiation of many narrowly distributed taxa, thus making wild carnations suitable models to study the evolutionary dynamics of plant species from temperate floras. Indeed, the study of the biological variation within species complexes belonging to the genus *Dianthus* allowed insights of many evolutionary dynamics, for example, polyploidization (Balao et al., 2009), phenotypic redundancy (Castro et al., 2022), and plasticity (Hardion et al., 2020). Hence, it is crucial to fill

gaps of knowledge concerning species complexes not only to better delimit taxa but also to gain new insights into the evolution of this genus.

The basic chromosome number $x = 15$ is constant in *Dianthus* and many species are diploid, albeit polyploidy is relatively common and tetraploids, pentaploids, hexaploids, and dodecaploids have been reported (Weiss et al., 2002; Balao et al., 2009; Rice et al., 2015; Terlević et al., 2022). Polyploidy in *Dianthus* is associated with genetic, phenotypic, physiological, and ecological diversification (Balao et al., 2010, 2011; López-Jurado et al., 2019; Domínguez-Delgado et al., 2021). On the other hand, some morphologically similar taxa can have different chromosome numbers (Jafari & Behroozian, 2010).

One of the most taxonomically critical complexes in the genus is that of *Dianthus virgineus* L. (Domina et al., 2021b), which includes 33 Euro-Mediterranean taxa (Marhold, 2011; Meyer, 2011; Tison & de Foucault, 2014; Bartolucci et al., 2018), growing in grasslands and rocky outcrops along a wide altitudinal range (Brullo & Guarino, 2017, 2019). In Southern-Central Europe, populations of this complex underwent a recent (200–115 kya) genetic divergence into three main allopatric lineages (Luqman et al., 2023). The populations from the Southern/Eastern Alps and from the Balkan Peninsula diverged earlier (178–217 kya) in a "Balkan" lineage, whereas the "Apennine" and the "Alpine" lineages, distributed in the westernmost Alps/Apennines and in the rest of the Alps, respectively, separated more recently (114–132 kya). In Central Mediterranean, where the "Apennine" lineage is widespread, morpho-ecological variation is currently organized in 21 phenotypically similar, mostly narrowly distributed, taxa (Bacchetta et al., 2010; Tison & de Foucault, 2014; Bartolucci et al., 2018). Nonetheless, these taxa were described mainly according to qualitative morphological variations and still await quantitative investigations. Hence, real biological differences among these taxa should be tested to properly circumscribe diversity groups in the *D. virgineus* complex. All Central Mediterranean species studied so far are diploid, with $2n = 30$ chromosomes (Rice et al., 2015; Bedini & Peruzzi, 2021). However, cytogenetic investigations of this complex are scarce, and karyological knowledge is lacking for 14 out of 21 taxa.

Cytotaxonomy focuses on studying metaphase chromosomes phenotype, such as their number, variation, and morphology, as these features directly influence plant evolution and speciation (Levin, 2002). The estimation of chromosome number, total haploid length (THL hereafter), and karyotype asymmetry indices provide useful insights to untangle systematic relationships within plant groups (Peruzzi & Altınordu, 2014; Astuti et al., 2017), especially when polyploidy/dysploidy is frequent (Peruzzi et al., 2009; Poggio et al., 2014; Winterfeld et al., 2020; Liu et al., 2022) or chromosomes are relatively large (Baeza et al., 2018; Turco et al., 2018; Goula et al., 2022; Tiburtini et al., 2022). However, when dealing with taxa showing a constant ploidy level and small chromosomes, karyological investigations can be less informative (Guerra, 2012). For instance, in *Dianthus*, karyomorphological features have often been neglected due to small chromosome size (0.5–2.5 μm), difficult detectability of centromeres, and slight interspecific karyotype differences (Behroozian et al., 2012; Şahin et al., 2016; Altay et al., 2017).

Another important cytogenetic parameter besides chromosome number and structure is genome size (GS hereafter), that is, the amount of cellular DNA contained in the nucleus (Pellicer et al., 2018). GS can be inferred using chromosome size as a gross proxy (Kramer et al., 2021) or experimentally assessed through flow cytometry (Doležel & Bartoš, 2005). Indeed, since GS is assumed to increase with ploidy level, flow cytometry is also used to detect polyploidization events (Suda et al., 2007b). Nevertheless, such inferences should be backed up by mitotic chromosome counting (Kolář et al., 2009; Frajman et al., 2018).

In the absence of polyploidy, GS can show a certain degree of variation within homoploid plant groups (Šmarda & Bureš, 2006; Loureiro et al., 2010; Frajman et al., 2015; Lazarević et al., 2015; Janišová et al., 2018), thus reflecting evolutionary dynamics not affected by genomic mutations (Pellicer et al., 2018). Neutral processes (Kang et al., 2014) and natural selection (Faizullah et al., 2021; Schley et al., 2022) can shape the amount of noncoding repetitive sequences in a genome, thus influencing GS variation (Macas et al., 2015; Hloušková et al., 2019). Hence, this variation may be associated with adaptation and diversification (Kraaijeveld, 2010; Trávníček et al., 2019; Carta et al., 2022b). Therefore, GS estimation can be considered a useful tool to investigate evolutionary relations among taxa (Suda et al., 2007a; Niketić et al., 2013; Terlević et al., 2022), especially if discrete changes in chromosome number and structure are absent (Loureiro et al., 2010).

Since patterns of diversity within the *D. virgineus* complex are poorly understood in the Central Mediterranean, in this work, we focused on the cytosystematics of 25 taxa of the complex including chromosome numbers, THL, and relative genome size (RGS hereafter) estimations. Specifically, we explored how chromosome number, THL, and RGS are related, and their variation among Central Mediterranean taxa and populations from the Alpine lineage (Terlević et al., 2022).

2 Material and Methods

2.1 Sampling

We sampled 122 populations belonging to 21 Central Mediterranean taxa, three Alpine taxa, and one cultivated species (~75% of taxa in the *Dianthus virgineus* complex) (Fig. 1A; Table S1): *Dianthus arrostoi* C.Presl, *Dianthus borbonicus* Brullo, C.Brullo, Colombo, Giusso, Ilardi & R.Perrone, *Dianthus brachycalyx* A.Huet & É.Huet ex Bacch., Brullo, Casti, & Giusso, *Dianthus busambrae* Soldano & F.Conti, *Dianthus caryophyllus* L., *Dianthus cyathophorus* Moris subsp. *cyathophorus*, *Dianthus cyathophorus* subsp. *minae* (Mazzola, Raimondo & Ilardi) Raimondo, *Dianthus gasparrinii* Guss., *Dianthus genargenteus* Bacch., Brullo, Casti, & Giusso, *Dianthus graminifolius* C.Presl, *Dianthus ichnusae* Bacch., Brullo, Casti, & Giusso subsp. *ichnusae*, *Dianthus ichnusae* subsp. *toddei* Bacch., Brullo, Casti, & Giusso, *Dianthus inodorus* (L.) Gaertn., *Dianthus insularis* Bacch., Brullo, Casti, & Giusso, *Dianthus japigicus* Bianco & Brullo, *Dianthus morisianus* Vals., *Dianthus mossanus* Bacch. & Brullo, *Dianthus oliastrae* Bacch., Brullo, Casti, & Giusso, *Dianthus sardous* Bacch., Brullo, Casti, & Giusso, *Dianthus saxicola* Jord., *Dianthus siculus* C.Presl, *Dianthus sylvestris* Wulfen s.l., *Dianthus tarentinus* Lacaita, *Dianthus*

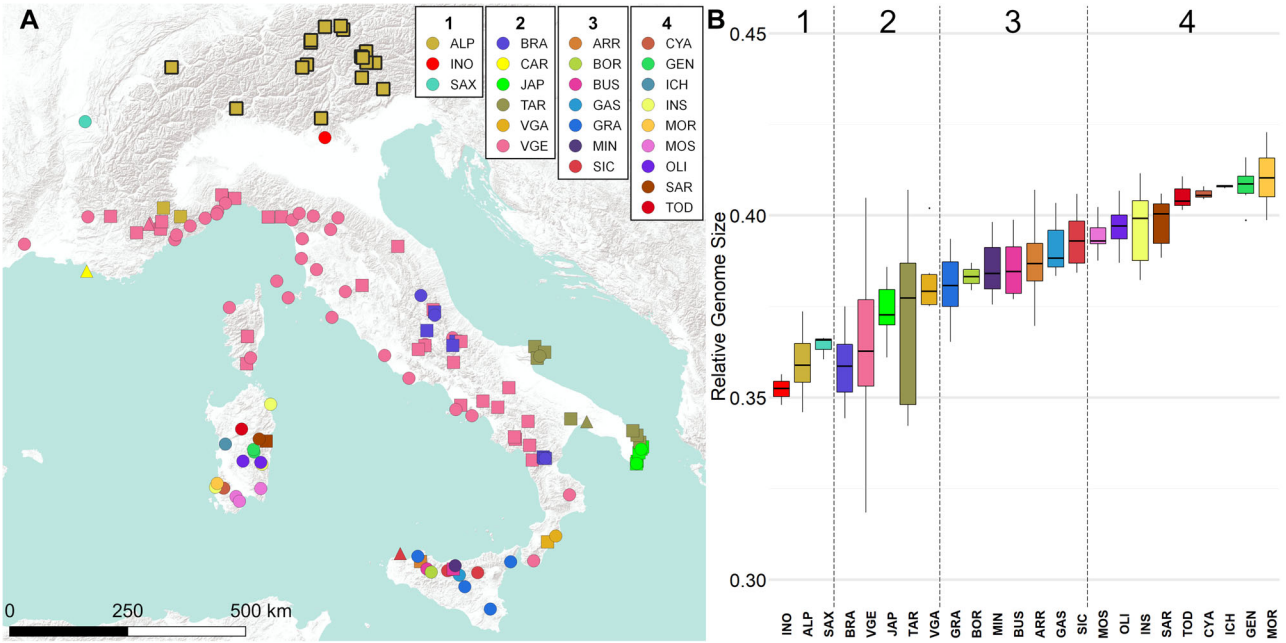

Fig. 1. Distribution of the studied populations and taxa of the *Dianthus virgineus* complex and their relative genome size (RGS) variation. **A**, Geographical distribution of the sampled populations. Circles represent populations for which both RGS and chromosome number were characterized ($n = 64$), squares represent populations for which only RGS was obtained ($n = 53$), while triangles represent populations for which only the chromosome number was obtained ($n = 5$). RGS data for populations represented by symbols with a bold outline were derived from Terlević et al. (2022). **B**, RGS variation among currently recognized taxa of the *D. virgineus* complex. Boxplots are arranged according to increasing median RGS value. Legends' headers: 1, taxa distributed across the Alps; 2, taxa spread across Southern France, Peninsular Italy, Tuscan Archipelago, and Corsica; 3, taxa occurring in Sicily; 4, taxa occurring in Sardinia. Colors correspond to different taxa. ALP, *Dianthus sylvestris* s.l.; ARR, *Dianthus arrostoi*; BOR, *Dianthus borbonicus*; BRA, *Dianthus brachycalyx*; BUS, *Dianthus busambrae*; CAR, *Dianthus caryophyllus*; CYA, *Dianthus cyathophorus* subsp. *cyathophorus*; GAS, *Dianthus gasparrinii*; GEN, *Dianthus genargenteus*; GRA, *Dianthus graminifolius*; ICH, *Dianthus ichnusae* subsp. *ichnusae*; INO, *Dianthus inodorus*, INS, *Dianthus insularis*; JAP, *Dianthus japigicus*; MIN, *Dianthus cyathophorus* subsp. *minae*; MOR, *Dianthus morisianus*; MOS, *Dianthus mossanus*; OLI, *Dianthus oliastrae*, SAR, *Dianthus sardous*; SAX, *Dianthus saxicola*; SIC, *Dianthus siculus*; TAR, *Dianthus tarentinus*; TOD, *Dianthus ichnusae* subsp. *toddei*; VGA, *Dianthus virgatus*; VGE, *D. virgineus*. The base map was provided by ESRI.

virgatus Pasq., and *D. virgineus*. Populations were selected to cover most of the range of the studied taxa and according to their taxonomic importance (e.g., populations from type localities were included). Taxonomic attribution of studied populations followed the treatment proposed by Bacchetta et al. (2010) for Southern-Central Italy, Sardinia, and Sicily, by also including taxa not directly treated in this revision (Bacchetta & Brullo, 2000; Raimondo et al., 2010; Brullo et al., 2015). For the rest of the study area, we considered as *D. virgineus* those populations recorded as *Dianthus godronianus* Jord. or *Dianthus longicaulis* Ten., as proposed by Domina et al. (2021b). Most of the Alpine populations were provisionally attributed to *D. sylvestris* s.l. (following Terlević et al., 2022), with the exception of the populations coming from the type localities of *D. inodorus* and *D. saxicola*, two species described from the Alps (Tison & de Foucault, 2014; Domina et al., 2021a, 2021b). A putatively naturalized population from Marseille (France) referred to *D. caryophyllus* (Tison et al., 2014), an ornamental species closely related to the *D. virgineus* complex (Domina et al., 2021b), was included in the study. We also collected material from Gorges de Daluis (France), where a tetraploid ($2n = 4x = 60$) cytotype of *D. virgineus* was reported (Löve, 1968; Tison et al., 2014). For 117 out of 122 populations, rosette leaves of individuals were collected and immediately put in silica gel for RGS estimation (Table S1). For 69 out of 122 populations, seeds were collected and germinated to obtain metaphase plates from root tips (Table S1), covering all studied taxa with the exception of *D. arrostoi*, a species distributed in Sicily, Calabria, and Northern Africa, for which we were not able to collect seeds. For 64 out of 122 populations, we were able to collect both silica-dried leaves and seeds (Table S1). For each sampled population, we provide the code of a voucher specimen deposited in public herbaria (Table S1).

2.2 Chromosome counts and THL measurement

Metaphase plates were obtained for 69 populations applying the Feulgen protocol as performed by Giacò et al. (2022). Seeds were incubated at diurnally alternating temperatures of 20 °C and 10 °C with 12 h of thermoperiod in darkness (Carta et al., 2022a). Root tips from germinated seeds were pretreated with 0.4% colchicine for 3 h and then fixed in Carnoy solution (3:1 ethanol:acetic acid) for 1 h at room temperature. After hydrolysis in 1 N HCl at 60 °C for 8.5 min, the tips were stained with leuco-basic fuchsine. Slides were prepared by chopping prepared tips with acetic orceine and observed with a Leitz Diaplan microscope. Images of the plates

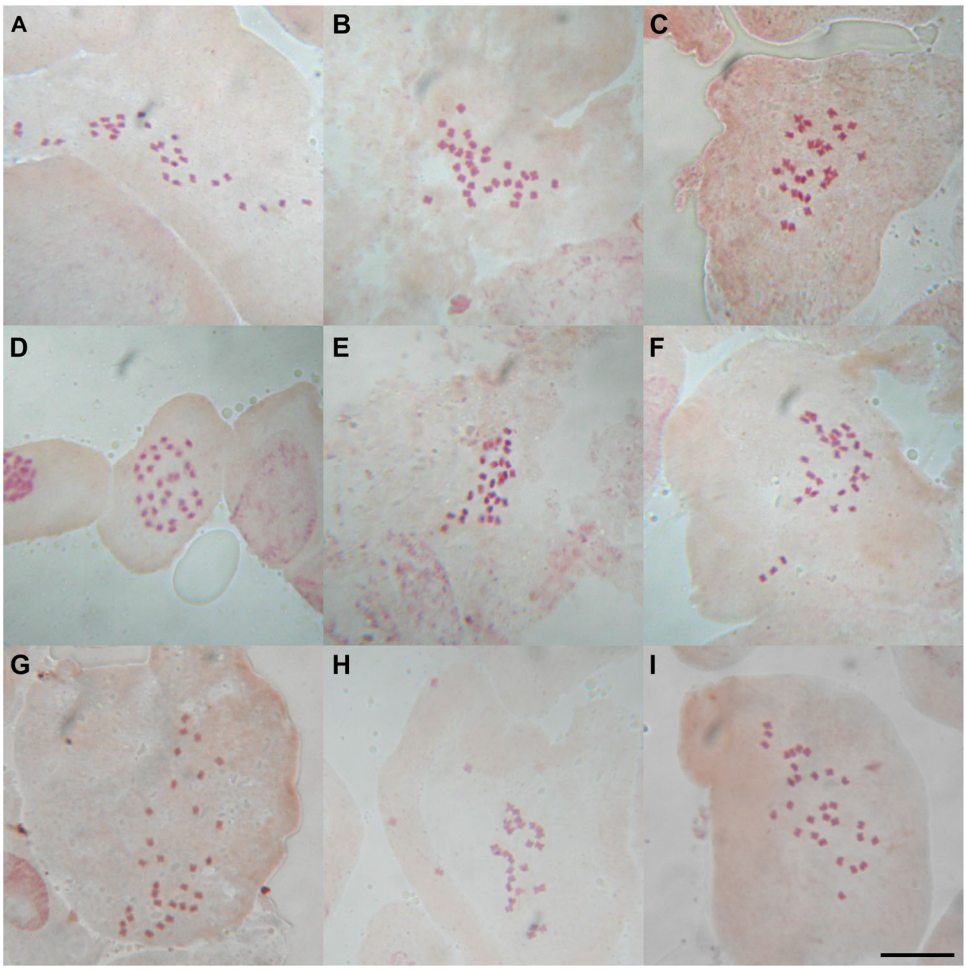

Fig. 2. Selected metaphase plates for taxa of the *Dianthus virgineus* complex distributed in the Alps, Southern France, Peninsular Italy, Tuscan Archipelago, and Corsica. **A**, *Dianthus brachycalyx* from Vado di Corno (Abruzzo, Italy). **B**, *Dianthus caryophyllus* from Cap Croisette (Provence-Alpes-Côte d′Azur, France). **C**, *Dianthus inodorus* from Busi di Avesa (Veneto, Italy). **D**, *Dianthus japigicus* from Torre Minervino (Puglia, Italy). **E**, *Dianthus saxicola* from Etang de Gillieu (Auvergne Rhône-Alpes, France). **F**, *Dianthus tarentinus* from Gravina di Leucaspide (Puglia, Italy). **G**, *Dianthus virgatus* from Stilo (Calabria, Italy). **H**, *Dianthus virgineus* from Montferrier sur Lez (Occitanie, France). **I**, *D. virgineus* from Gorges de Daluis (Provence-Alpes-Côte d′Azur, France). Scale bar, 10 μm.

were acquired using a Canon PowerShot S45 camera. The number of metaphase plates used to establish the chromosome number per population is reported in Table S1. THL of the best-looking metaphasic plates, showing similar degrees of DNA condensation, was calculated for 64 populations (those for which both RGS and THL could be estimated) using the software KaryoType (Altınordu et al., 2016).

2.3 RGS estimations

RGS was estimated for 357 individuals from 117 populations using flow cytometry as described by Suda & Trávníček (2006), with modifications as in Terlević et al. (2022). We mainly performed RGS estimations using silica-dried leaves. Although the use of fresh leaves is preferable when performing flow cytometry, the use of dehydrated leaves has also been shown to be effective (Suda & Trávníček, 2006). Indeed, there is a plethora of published works fruitfully estimating RGS using dried material (e.g., Kolář et al., 2013; Kutnjak et al., 2014; Cresti et al., 2019; Frajman et al., 2019; Caković et al., 2021). The number of individuals used for each population is reported in Table S1. Briefly, silica-dried leaves of each studied individual were co-chopped with fresh leaves of *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al., 2007) used as a reference standard. After extraction, nuclei were stained with 0.036 M 4′,6-diamidino-2-phenylindole (DAPI) and the relative fluorescence intensity of 3000 nuclei was recorded using a Partec CyFlow Space flow cytometer. Using Partec FloMax software, histograms of fluorescence intensity ("peaks" hereafter) were obtained and coefficients of variation (CVs) and RGS of our samples were calculated for each individual in terms of arbitrary units ("a.u." hereafter). Since dried material contains lower-quality nuclei compared to fresh leaves, thus retrieving more dispersed peaks (higher CVs), peaks with CV < 10% were retained for downstream analyses. To test the accuracy of the RGS estimations performed with silica-dried leaves, we also assessed RGS using freshly collected leaves from a subset of populations, geographically representative of the study area. In addition,

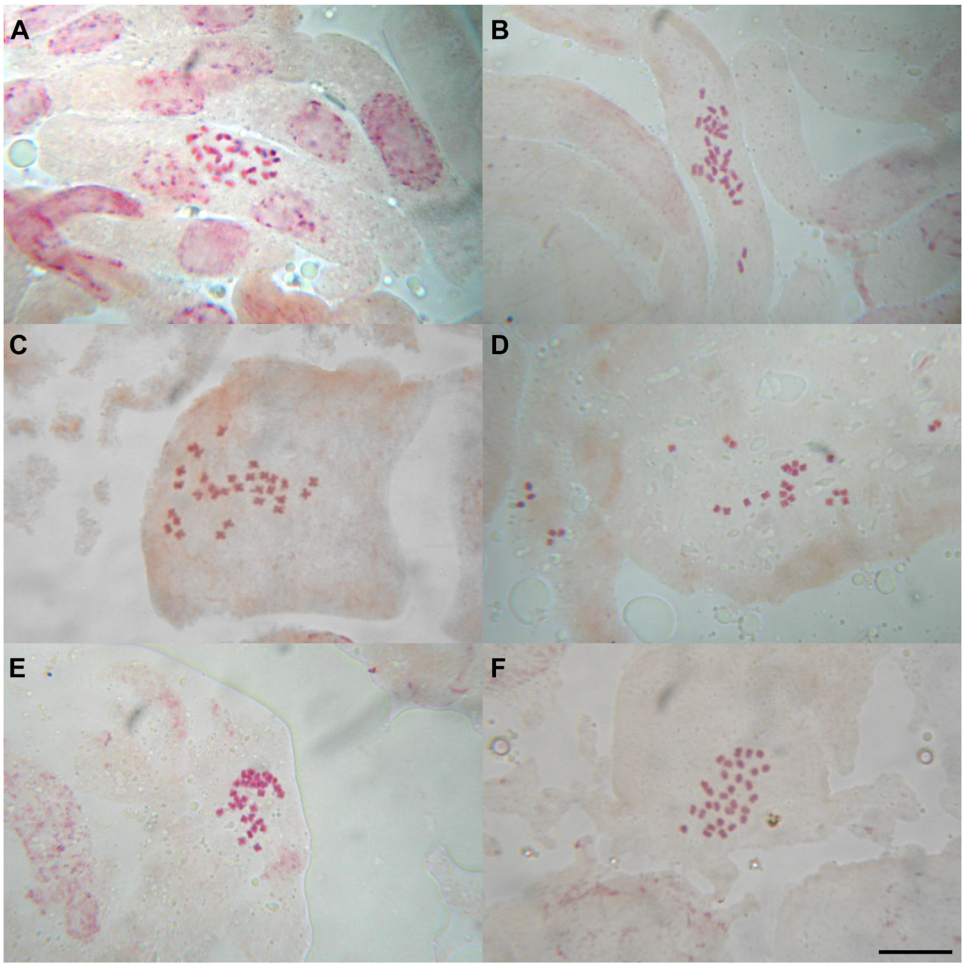

Fig. 3. Selected metaphase plates for the Sicilian taxa of the *Dianthus virgineus* complex. **A**, *Dianthus borbonicus* from Pizzo Morabito. **B**, *Dianthus busambrae* from Rocca Busambra. **C**, *Dianthus gasparrinii* from Petralia Sottana. **D**, *Dianthus graminifolius* from Montelepre. **E**, *Dianthus cyathophorus* subsp. *minae* from Passo Scuro. **F**, *Dianthus siculus* from Monte Passo del Lupo. Scale bar, 10 μm.

RGS data for 17 Alpine populations were obtained from Terlević et al. (2022).

2.4 Statistical analyses

Data visualization and analysis were performed using QGIS v. 3.26.3 and R (R Core Team, 2020). Since chromosome numbers were the same for all the studied populations, this parameter was not further statistically explored. Univariate analyses were performed to test significant differences of RGS among taxonomic and geographical groups. To check if chromosome length may be related to the RGS of the studied populations, Spearman's correlation test between THL and RGS was performed.

We first assessed significant differences of RGS among currently recognized taxa. Then, irrespective of the current taxonomy, we grouped the populations into four geographical groups (GGs hereafter), based on their distribution across the study area, and tested significant RGS differences among them. The first GG contained the accessions from the Alps, which belong to a genetically divergent lineage (Luqman et al., 2023) (ALP; 28 individuals). The second GG included individuals from Southern France and Peninsular Italy (PEN; 250 individuals), most of which belong to the "Apennine" lineage (Luqman et al., 2023). Insular populations were assigned to two further GG, one containing individuals from Sicily (SIC; 32 individuals) and the other from Sardinia, Corsica, and Tuscan Archipelago (SAR; 66 individuals), given the biogeographical affinity among these islands (Thompson, 2020). Since both taxa and GGs were heteroskedastic (Bartlett test, $P < 0.0001$) with different sample sizes, RGS differences were tested using Welch's analysis of variance (ANOVA) and Games–Howell post hoc test (Games & Howell, 1976; Kassambara, 2021). Effect size was estimated using Cohen's *d* (Cohen, 1988). We also tested for the correlation between RGS and latitude within the PEN GG, which showed high RGS variability, using Spearman's test and the data were interpolated with a quadratic regression line.

3 Results

3.1 Chromosome counts and THL

All 69 studied populations, referring to all the studied taxa, had $2n = 30$ chromosomes. Metaphase plates are shown in

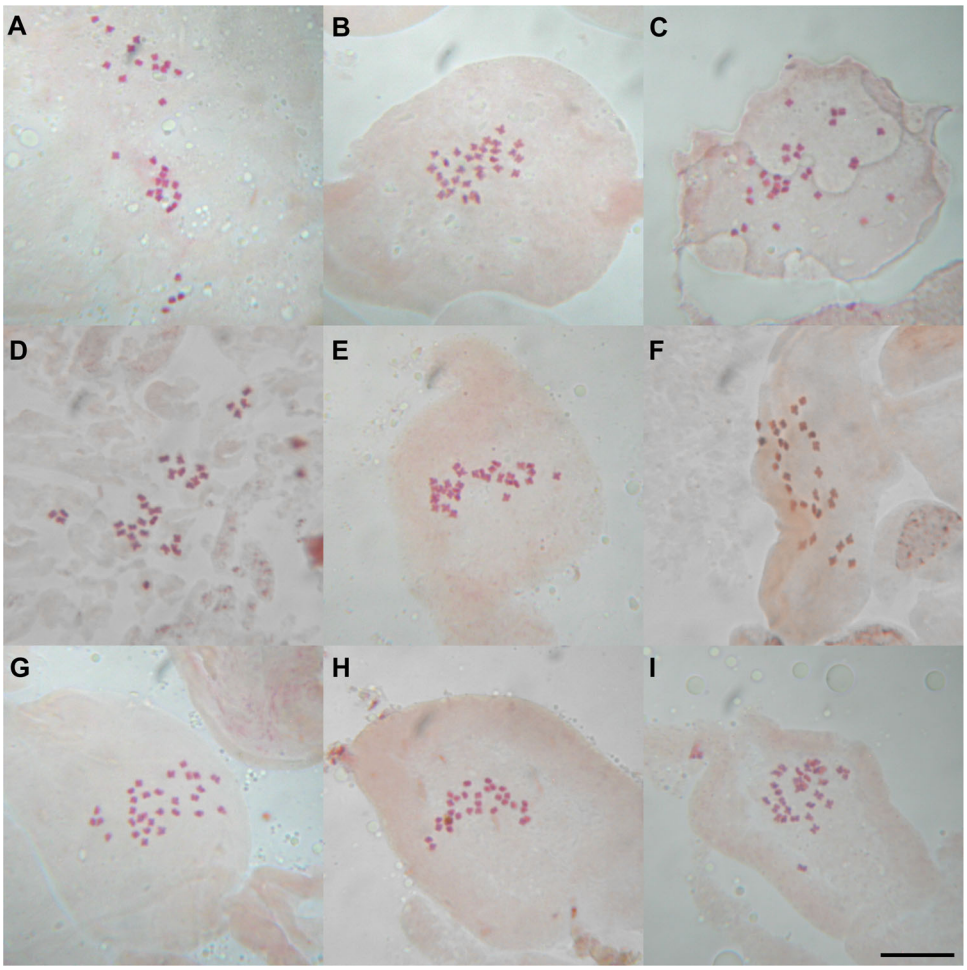

Fig. 4. Selected metaphase plates for the Sardinian taxa of the *Dianthus virgineus* complex. **A**, *Dianthus cyathophorus* subsp. *cyathophorus* from Foresta di Marganai. **B**, *Dianthus genargenteus* from Bruncu Spina-Desolu. **C**, *Dianthus ichnusae* subsp. *ichnusae* from Montiferru. **D**, *Dianthus insularis* from Tacco di Ulassai. **E**, *Dianthus morisianus* from Portixeddu. **F**, *Dianthus mossanus* from S'Enna Sa Craba. **G**, *Dianthus oliastrae* from Laconi. **H**, *Dianthus sardous* from Monte Corrasi. **I**, *Dianthus ichnusae* subsp. *toddei* from Monte Rasu. Scale bar, 10 μm.

Figs. 2–4. Here, we have reported for the first time the chromosome number for 14 taxa of the complex (Table 1). The THL of 63 out of 64 studied populations ranged from 14.09 to 20.71 μm (mean ± SD = 17.45 ± 1.63 μm) (Table S1). One outlying measurement (THL = 24.32 μm) was removed because chromosomes clearly showed a lower degree of condensation. No significant correlation was found between THL and RGS variation ($r_s = -0.078$, $P = 0.543$) (Fig. S1).

3.2 RGS variation

The RGS of 357 individuals from 117 populations ranged between 0.318 and 0.423 a.u. The mean CV of the fluorescence peaks of our samples was 4.78% (min 1.96%, max 12.03%, SD 1.76; Table S1). For three accessions, the CV was slightly over 10%, but the sample peaks were easily recognizable and RGS values were in agreement with other values from the same population. Estimations performed on fresh leaves were in agreement with the ones obtained from dehydrated material (Table S2). RGS data were in line with the chromosome counts, as no polyploidy was detected. Despite all individuals being RGS-diploid, we observed a certain degree of RGS variation (mean = 0.373 a.u., SD = 0.019), with a wide overlap in RGS among different taxa (Fig. 1B). Out of the 276 pairwise comparisons to test differences of RGS among 24 taxa, only 67 were significant ($P < 0.01$). Most of these differences (55 out of 67) were detected when comparing RGS between Sardinian taxa and others (Table S3). Additionally, the highest effect size was obtained when confronting Sardinian taxa with those from elsewhere in the study area (Table S3). By ignoring current taxonomic treatments and considering the populations clustered in GGs, significant RGS differences were observed among ALP, PEN, SIC, and SAR groups (ANOVA, $F = 130.07$, $P < 0.0001$) (Figs. 5A, 5B; Table 2). SAR showed higher RGS values with respect to other GGs, whereas ALP had a lower value (Figs. 5A, 5B; Table 2). RGS of PEN did not vary linearly with latitude ($r_s = 0.039$, $P = 0.736$), but decreased from the Mediterranean France and North-Western Italy (Liguria) to Southern-Central Italy, and increased again in Southern Italy (Calabria and Puglia; quadratic regression, $R^2 = 0.273$, $F = 15.48$, $df = 2, 75$, $P < 0.0001$; Fig. 5C). An example of a good-quality peak obtained from silica-dried leaves is shown in Fig. 6A (more fluorescence histograms are presented in Figs. S2–S4).

Table 1 Taxa belonging to the *Dianthus virgineus* complex in the Central Mediterranean, whose chromosome number is presented here for the first time

Taxon	2*n*
Dianthus borbonicus Brullo, C.Brullo, Colombo, Giusso, Ilardi & R.Perrone	30
Dianthus brachycalyx A.Huet & É.Huet ex Bacch., Brullo, Casti & Giusso	30
Dianthus busambrae Soldano & F.Conti	30
Dianthus cyathophorus Moris subsp. *minae* (Mazzola, Raimondo & Ilardi) Raimondo	30
Dianthus gasparrinii Guss.	30
Dianthus genargenteus Bacch., Brullo, Casti & Giusso	30
Dianthus graminifolius C.Presl	30
Dianthus ichnusae Bacch., Brullo, Casti & Giusso subsp. *ichnusae*	30
Dianthus ichnusae subsp. *toddei* Bacch., Brullo, Casti & Giusso	30
Dianthus insularis Bacch., Brullo, Casti & Giusso	30
Dianthus oliastrae Bacch., Brullo, Casti & Giusso	30
Dianthus saxicola Jord.	30
Dianthus tarentinus Lacaita	30
Dianthus virgatus Pasq.	30

For each taxon, the chromosome number (2*n*) is reported.

Separated peaks were retrieved on analyzing simultaneously samples showing different RGS values, either belonging to different groups (Fig. 6B) or to the same GG (Fig. 6C).

4 Discussion

4.1 Ploidy level of Central Mediterranean taxa of the *Dianthus virgineus* complex

Our chromosome counts and RGS estimations indicated absence of variation in chromosome number and ploidy level among taxa belonging to the *Dianthus virgineus* complex in the Central Mediterranean. Although we did not find chromosomal changes among the studied populations, we filled gaps of karyological knowledge for 14 taxa, for which there was no information available concerning ploidy level or chromosome number. The obtained chromosome counts are in agreement with those available in the literature for the other taxa belonging to the *D. virgineus* complex in Italy (Bedini & Peruzzi, 2021) and France (Rice et al., 2015). Although we were not able to determine the chromosome number of *Dianthus arrostoi*, this species was already known to be diploid with $2n = 30$ chromosomes (Raimondo et al., 1983). The absence of chromosome number variation in the *D. virgineus* complex stands in contrast to other European species groups of *Dianthus* (Weiss et al., 2002; Balao et al., 2009), while in the "Balkan" lineage, only localized tetraploidization was inferred in the north-westernmost portion of the peninsula (Terlević et al., 2022). Some of the published putatively polyploid chromosome counts (Darlington & Wylie, 1956; Löve, 1968) may not be reliable. In fact, putatively tetraploid individuals previously reported from Gorges de Daluis (Maritime Alps, France; Löve, 1968) were not confirmed in this study, as our samples from this locality were clearly diploid (Fig. 2). This discrepancy could be due to the possible presence of endoreduplication in root tips, which is a chromosome duplication that occurs without decondensation and condensation of DNA (Joubès & Chevalier, 2000). This phenomenon has already been documented in carnations (Agulló-Antón et al., 2013), and we actually found single cells showing more than 30 chromosomes in root tips of seeds collected from Gorges de Daluis, but also from other populations (Fig. S5). Accordingly, the hexaploid status of the Iberian *Dianthus boissieri* Willk., also belonging to the *D. virgineus* complex, should also be verified (Darlington & Wylie, 1956; Bernal et al., 1990).

4.2 Patterns of RGS variation of the *Dianthus virgineus* complex in the Central Mediterranean

To compare the observed GS variation with other groups of *Dianthus* taxa, we calculated the coefficient of GS variation (CV_{GS} hereafter). GS of the studied individuals from Central Mediterranean varies considerably ($CV_{GS} = 5.24\%$) if compared to the GS variation of the Alpine populations ($CV_{GS} = 1.94\%$), to diploid populations from the "Balkan" lineage ($CV_{GS} = 3.30\%$; Terlević et al., 2022), or to diploids belonging to *Dianthus broteroi* Boiss. & Reut. from Southern Spain ($CV_{GS} = 3.56\%$; Balao et al., 2009). RGS data interpretation is limited by the use of dehydrated leaves. However, such a consistent variation cannot be explained by an overestimation of RGS due to low-quality nuclei extracted from dried leaves, since the estimations that we performed on fresh material are fully congruent with those obtained from dehydrated leaves (Table S2). One may argue that since DAPI is an AT-selective fluorescent dye (Doležel et al., 1992), the observed variation in RGS may be biased by a different GC content among the genomes of the studied individuals. However, the proportion of GC in a genome varies only slightly within a family (Barow & Meister, 2002), so that different fluorescence responses mirror actual GS variations rather than differences in the GC content at low taxonomic levels (Kolář et al., 2013). Thus, our data reflect a genuinely high degree of variation in GS of the *Dianthus virgineus* complex in the Central Mediterranean with respect to other diploid *Dianthus* groups.

The assessed RGS variation is not in agreement with current taxonomic treatment within the *D. virgineus* complex in the Central Mediterranean. For instance, morphologically and ecologically similar taxa, like *Dianthus brachycalyx* and *Dianthus ichnusae* subsp. *ichnusae* (Bacchetta et al., 2010), show significantly different RGS values and are geographically well separated, as the former is endemic to Southern-Central Peninsular Italy and the latter to Sardinia. On the other hand, morphologically distinct but geographically close taxa, like *Dianthus cyathophorus* subsp. *cyathophorus* and *Dianthus genargenteus* (Bacchetta et al., 2010), show no RGS differentiation. Contrary to RGS differences revealed among some taxa endemic to small areas, RGS of more widespread species like *D. virgineus* spans most of the RGS variation of other taxa.

Overall, the RGS variation among the studied populations follows a geographical pattern. RGS varies significantly among the Alpine region, Southern France and Peninsular Italy, Sicily, and the Corso-Sardinian system (including the Tuscan Archipelago). This variation may reflect genetic divergences, as, for instance, Alpine populations (ALP) have

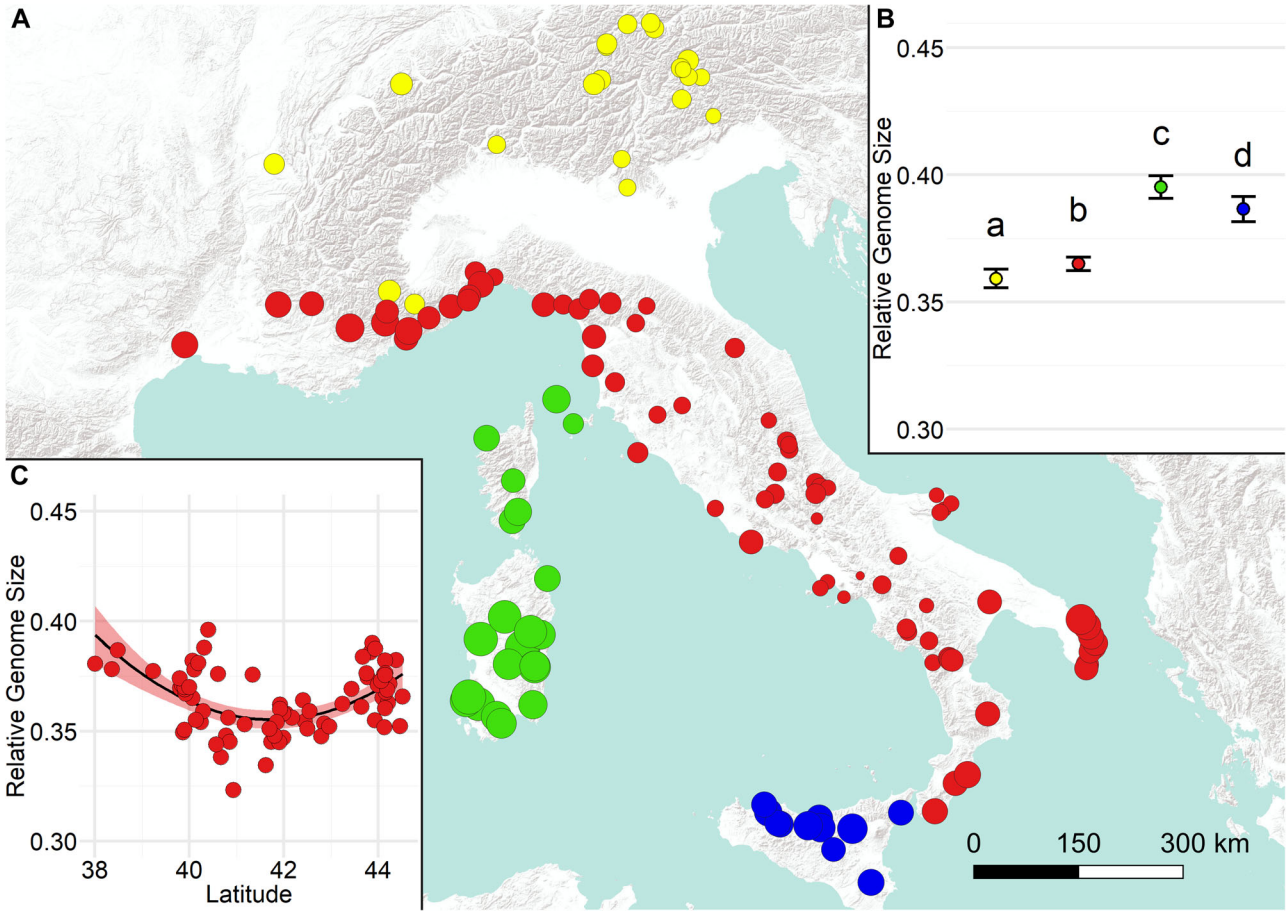

Fig. 5. Geographical patterns of relative genome size (RGS) variation in the *Dianthus virgineus* complex from the Central Mediterranean and the Alps. **A**, Mean RGS values of each studied population. Circles size is proportional to RGS. **B**, Mean RGS value and 99% confidence interval for each geographical group. Letters above symbols indicate significant differences. **C**, Quadratic relation between latitudinal variation and RGS across PEN populations ($R^2 = 0.273$, $F = 15.48$, $df = 2, 75$, $P < 0.0001$). The 95% confidence interval of the regression line is shown. Yellow: populations from the "Alpine" lineage (ALP); red: populations from Southern France and the Italian Peninsula (PEN); green: populations from Sardinia, Corsica, and Tuscan Archipelago (SAR); and blue: populations from Sicily (SIC). The base map was provided by ESRI.

Table 2 Significant RGS differences among geographical groups (GGs) in the *Dianthus virgineus* complex

	ALP	PEN	SAR	SIC
ALP				
PEN	$P < 0.01$			
	$d = 0.38$			
SAR	$P < 0.0001$	$P < 0.0001$		
	$d = 2.98$	$d = 1.94$		
SIC	$P < 0.0001$	$P < 0.0001$	$P < 0.01$	
	$d = 3.08$	$d = 1.41$	$d = 0.67$	

Each cell represents a pairwise comparison between GGs. For each comparison, significance and Cohen's *d* are shown; ALP, GG containing individuals from the Alps belonging to the "Alpine" lineage (28 individuals); PEN, GG containing individuals from Southern France and Peninsular Italy (250 individuals); SAR, GG containing individuals from Sardinia, Corsica, and Tuscan Archipelago (66 individuals); SIC, GG containing individuals from Sicily (32 individuals).

a smaller RGS than those from Southern France and Peninsular Italy ("Apennine" lineage; Luqman et al., 2023). In addition, insular populations have larger RGS than those from Peninsular Italy and Southern France. We may hypothesize that geographic isolation, following recent paleogeographical events (Thompson, 2020) of populations from Sicily and the Corso-Sardinian system, promoted genetic drift and/or local selection, thus maintaining an increased RGS. Upcoming genomic data will be vital to understand if the RGS variation is congruent with the genetic diversification of the studied populations.

The RGS cline observed across Southern France and Italian Peninsula may suggest diversification phenomena in the absence of relevant geographical barriers. This cline somewhat mirrors the RGS variation among diploid populations of *Dianthus sylvestris* from the Balkan Peninsula, where increasing RGS toward latitudinal extremes of the species range has been observed (Terlević et al., 2022). Even though such a pattern has also been found in other European plant groups (*Knautia* L., Frajman et al., 2015; *Sesleria* Scop.,

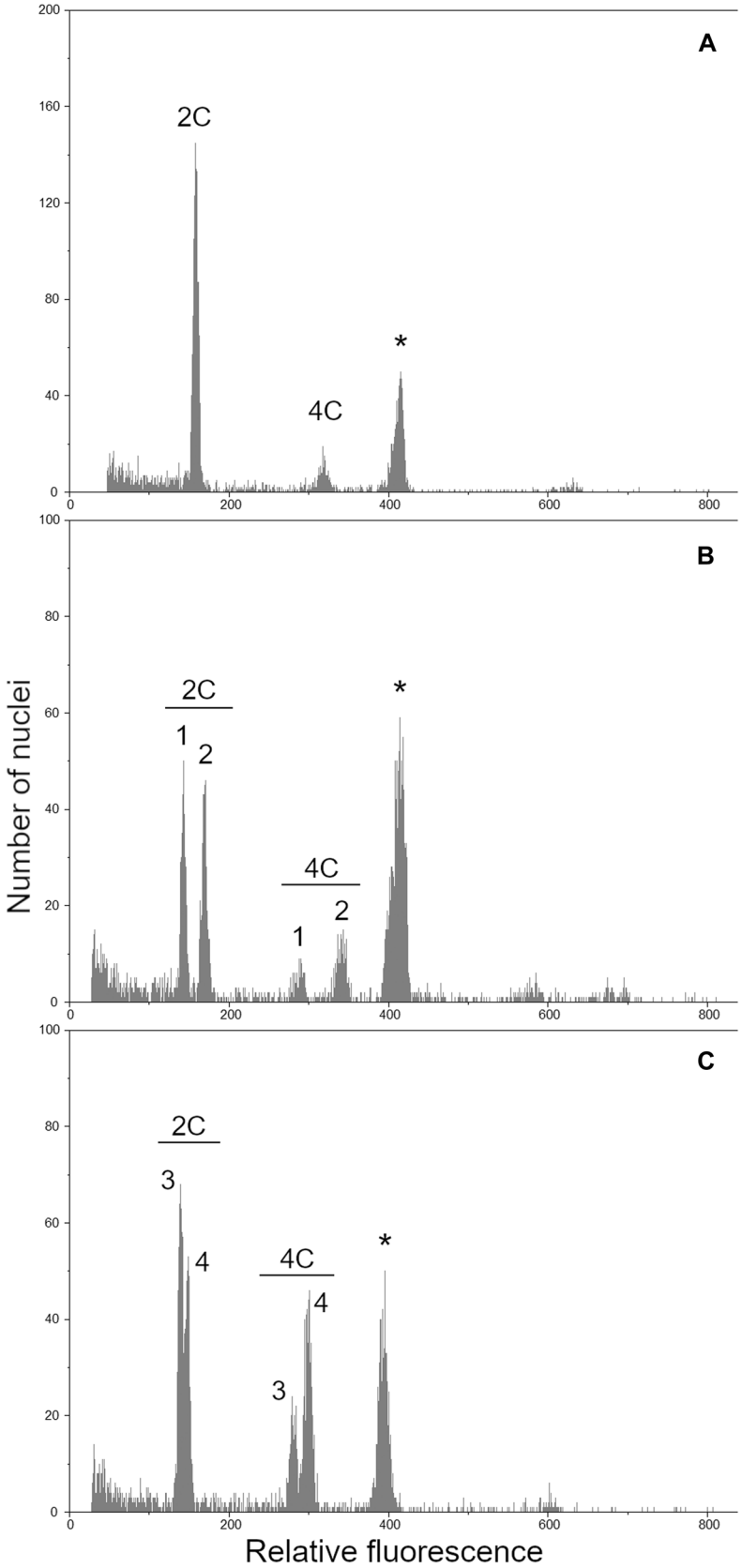

Fig. 6. Continued

Lazarević et al., 2015), only speculations on its causes can be made. It has been proposed that larger genomes may be disadvantageous in terms of adaptation and competition in peripheral portions of the range, further limiting its expansion (Knight, 2005). In our study system, such a scenario would suggest a continuous expansion from a single Pleistocene refugial area in Southern-Central Italy, where we detected the lowest RGS values. However, we cannot exclude the existence of different refugia correlated with different RGS across the Italian Peninsula, which would be in line with the "refugia-within-refugia" hypothesis (Nieto Feliner, 2014).

The obtained RGS values support the distinctiveness of the "Apennine" and the "Balkan" lineages *sensu* Luqman et al. (2023). The populations from the "Apennine" lineage are characterized by higher RGS (mean ± SD = 0.374 a.u. ± 0.019) compared to the diploids from the "Balkan" lineage (mean ± SD = 0.341 a.u. ± 0.011; Terlević et al., 2022). A similar pattern was detected in diploid populations of *Knautia* subg. *Trichera* (Schrad.) Rouy, where Balkan populations had smaller GS compared to those from the Apennines (Frajman et al., 2015). Overall, the RGS variation within the *D. virgineus* complex across the Alpine, Apennine, and Balkan regions parallels the temporal sequence of divergences among the three genetic lineages (Luqman et al., 2023), which have also been shown to be morphologically differentiated (Gargano et al., 2023). Early divergent "Balkan" lineage has smaller genome compared to the "Alpine" and the "Apennine" lineages. On the other hand, less conspicuous differences in RGS were detected between more closely related "Alpine" and "Apennine" lineages. The RGS differences may result from persistence of the three genetic groups in isolated glacial refugia that triggered RGS divergence, as nowadays, they show allopatric distributions. Independent diversification dynamics of the three lineages have been proposed also by Luqman et al. (2023).

4.3 THL and RGS relation

Even though THL is reported to be a suitable proxy for estimating GS in some plant groups (Carta & Peruzzi, 2016; Kramer et al., 2021), no correlation between THL and RGS was found in the *Dianthus virgineus* complex. Probably, the stable chromosome number and the small size of chromosomes, well documented in this genus (Şahin et al., 2016; Altay et al., 2017), may hamper the detectability of the correlation between GS and chromosome length. A different degree of chromatin condensation among studied metaphase plates is known to significantly influence estimations of karyotype features (Guerra, 2012). However, when chromosomes are small, such changes would not bias results as much as in large-sized chromosomes species (e.g., Miranda et al., 2007). The use of other indices to estimate chromosome size may allow detection of the correlation between karyotype size and RGS. For instance, measuring chromosome volume instead of length (De Vescovi & Sziklai, 1975) may yield clearer results when correlating GS and chromosome size. This parameter is known to covariate with GS on a macroevolutionary level, for example, in vertebrates (Kramer et al., 2021). In plants with small chromosomes, chromosome volume could show a stronger relationship with GS than chromosome length (Mehravi et al., 2022).

5 Conclusions

A constant chromosome number and diploid level, coupled with few RGS differences, were observed among taxa of the *Dianthus virgineus* complex in the Central Mediterranean. Our results support previous chromosome counts by also providing new information for 14 taxa of the complex. A geographical, rather than taxonomic, RGS variation allowed speculations on the actual evolutionary relationships within this complex. For instance, a number of evolutionary unique groups lower than the number of currently accepted taxa may be hypothesized, pending integration with new morphometric and genomic data. THL was not able to reflect small variations of RGS, whereas flow cytometry-mediated RGS estimation unraveled patterns of variation at different geographical and evolutionary scales.

Acknowledgements

This work was supported by the "Progetto di Ricerca di Rilevante Interesse Nazionale" (PRIN) "PLAN.T.S. 2.0—towards a renaissance of PLANt Taxonomy and Systematics" led by the University of Pisa, under the grant number 2017JW4HZK (Principal Investigator: Lorenzo Peruzzi), by the Croatian Science Foundation under the project UIP-2017-05-2882 (AmphiAdriPlant), and the Austrian Agency for International Cooperation and the Croatian Ministry of Science and Education (Austria-Croatia bi-lateral project "HR17/2020" to Božo Frajman). We are grateful to Marianne Magauer, Barbara Pernfuß, and Daniela Pirkebner for practical support during lab activities. We also thank Sandro Bogdanović, Chiara Bonifazio, Paola De Giorgi, Salvatore Cambria, Angelino Congiu, Giuseppe Fenu, Antonio Giacò, Mario Pappacoda, Michele Innangi, Matthew Chidozie Ogwu, Pasqualino Paris, Lina Podda, Marco Porceddu, Ivana Rešetnik, Danila Scalzo, Martina Temunović, Manuel Tiburtini, and Valeria Tomaselli for their help during sampling and Giovanni Gestri, Lorenzo Pinzani, Jean-Marc Tison, and Daniele Viciani for providing information on some of the studied populations.

Fig. 6. Fluorescence intensity histograms obtained from samples belonging to the *Dianthus virgineus* complex showing 2C, 4C, and standard fluorescence peaks (*). **A**, histogram showing a peak obtained from a silica-dried sample of *D. virgineus* collected from Montferrier sur Lez (Occitanie, France). **B**, Histograms showing distinct peaks of samples belonging to different geographical groups and characterized by different genome size values; 1, sample of *Dianthus inodorus* from Busi di Avesa (Veneto, Italy), belonging to ALP; 2, sample of *Dianthus morisianus* from Portixeddu (Sardinia, Italy), belonging to SAR. **C**, histograms showing distinct peaks of sample belonging to PEN that are characterized by different RGS values; 3, sample of *Dianthus brachycalyx* from Vado di Corno (Abruzzo, Italy); 4, sample of *Dianthus virgatus* from Stilo (Calabria, Italy).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Accessibility Statement

FCS files are openly available in Figshare digital repository (doi:10.6084/m9.figshare.23553249).

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.13025/suppinfo:

Fig. S1. Relative genome size (RGS) and total haploid length (THL) variation in *Dianthus virgineus* complex from the Central Mediterranean and the Alps.

Fig. S2. Histogram of fluorescence intensity of nuclei extracted from silica-dried leaves of individuals belonging to taxa of the *Dianthus virgineus* complex distributed in the Alps, Southern France, Peninsular Italy, Tuscan Archipelago, and Corsica.

Fig. S3. Histogram of fluorescence intensity of nuclei extracted from silica-dried leaves of individuals belonging to Sicilian taxa of the *Dianthus virgineus* complex.

Fig. S4. Histogram of fluorescence intensity of nuclei extracted from silica-dried leaves of individuals belonging to Sardinian taxa of the *Dianthus virgineus* complex.

Fig. S5. Endoreduplication in root tips of the *Dianthus virgineus* complex.

Table S1. Karyological and geographical information of characterized populations belonging to the *Dianthus virgineus* complex.

Table S2. Comparison between relative genome size (RGS) values obtained using silica dried and fresh leaves of the same populations within the *Dianthus virgineus* complex in the Central Mediterranean.

Table S3. Significant relative genome size (RGS) differences among current taxonomic hypotheses of the *Dianthus virgineus* complex.