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# Diploids and polyploids in the *Santolina chamaecyparissus* complex (Asteraceae) show different karyotype asymmetry

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**ABSTRACT**

A traditional karyomorphological approach was carried out in order to investigate the cytotaxonomic relationships among the species of the *Santolina chamaecyparissus* complex. All the 14 species included in this species complex were analysed for a total of 39 populations. Metaphase plates were obtained by squashing root tips stained with Feulgen technique. Measurable metaphase plates available in literature were also re-analysed. Chromosome number (2*n*), total haploid (monoploid) length (THL), and karyotype asymmetry indices ($CV_{CL}$ and $M_{CA}$) were calculated for each of the 14 species. Chromosome data are provided for the first time for *S. benthamiana* and *S. vedranensis*, while a new hexaploid cytotype is reported for *S. villosa*. Five different ploidy levels are detected: diploid, triploid (a single individual of the otherwise diploid *S. virens*), tetraploid, pentaploid, and hexaploid. Our analyses reveal that polyploids are characterized by a more asymmetric karyotype with respect to diploids. The possible origin of polyploids, and in particular of the cultivated pentaploid *S. chamaecyparissus*, is briefly discussed.

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Cytogenetics; karyotype; taxonomy; systematics; Mediterranean Basin

## Introduction

The *Santolina chamaecyparissus* complex includes 14 species that are endemic to the western portion of the Mediterranean Basin (Greuter 2008). These plants are dwarf aromatic shrubs occurring in arid habitats and preferably on limestone, where they usually form garigues (Arrigoni 1982a; Angiolini and Bacchetta 2003; Carbajal et al. 2019). Four different ploidy levels are currently known in this complex. The continental species, except *S. villosa* Mill. that is reported as tetraploid ($2n = 4x = 36$), are diploid with $2x = 2n = 18$ chromosomes (Arrigoni 1977; Valdés-Bermejo and Antúnez 1981; Kyohs 1982; Afzal-Rafii et al. 1985). *Santolina corsica* Jord. & Fourr., a species endemic to Corsica and Sardinia, is tetraploid, and *S. ins*ularis (Gennari ex Fiori) Arrigoni, which is endemic to Sardinia only, is hexaploid ($2n = 6x = 54$) (Marchi and D'Amato 1973; Marchi et al. 1979). *Santolina chamaecyparissus* L., the nomenclatural type of the genus *Santolina* L. (Greuter 2008), is pentaploid with $2n = 5x = 45$ chromosomes (Marchi and D'Amato 1973). The latter is a commonly cultivated species of unknown origin, very appreciated as ornamental in gardens and hedges (Giacò et al. 2021). According to Marchi and D'Amato (1973), it may have been originated by a crossing between *S. insularis* and another tetraploid *Santolina*. According to qualitative cytogenetic studies, polyploid species of the *S. chamaecyparissus* complex seemingly show a higher number of subterminal chromosomes (Marchi and D'Amato 1973; Marchi et al. 1979; Valdés-Bermejo and Antúnez 1981). Previous studies on the *S. rosmarinifolia* L. complex (Rivero-Guerra 2008a, 2008b, 2010) highlighted that, in Iberian Peninsula, polyploidy and chromosome rearrangements played an important role in the segregation of taxa. Based on the current knowledge, we hypothesize that these phenomena played an important role in the evolution of the *S. chamaecyparissus* complex as well. In order to investigate the diversity at a cytogenetic level among the species of this complex, we carried out a karyomorphological study (Paszko 2006; Peruzzi and Eroğlu 2013; Peruzzi and Altınordu 2014).

Karyomorphology, i.e. the study of the general morphology of a chromosome complement, is a cheap and relatively easy method to investigate plant diversity at different taxonomic levels (Peruzzi et al. 2009; Astuti et al. 2017). With Feulgen or similar staining techniques, the most common studied parameters are the chromosome number (2*n*), the basic chromosome number (*x*), the Total Haploid (monoploid)

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Length (THL), and the karyotype asymmetry (Astuti et al. 2017). The first two parameters provide basic and fundamental information to understand relationships among taxa, especially when studying groups showing dysploidy and/or polyploidy (Levin 2002). THL is a good proxy for genome size (Peruzzi et al. 2009; Carta and Peruzzi 2016). Karyotype asymmetry depends on the heterogeneity of chromosome sizes in a complement (inter-chromosomal asymmetry), and on the deviation degree of centromeres from the median position in each chromosome (intra-chromosomal asymmetry) (Levitsky 1931; Stebbins 1971). Albeit the limitations of the traditional karyomorphological approach are well known (Guerra 2012; Astuti et al. 2017), it can provide valuable information to better understand evolutionary trends and to infer taxonomic relationships, especially when integrated with other approaches, such as molecular and morphological analyses (Caputo et al. 2013; Nardi et al. 2018; Bernardo et al. 2020; Ghosh et al. 2021).

The aim of this study is to have a complete picture about the chromosome number and karyotype structure of the species belonging to *S. chamaecyparissus* complex. Moreover, we want to quantitatively check if there are significant karyomorphological differences among diploid and polyploid karyotypes, as suggested by previous scholars (Marchi and D'Amato 1973; Marchi et al. 1979; Valdés-Bermejo and Antúnez 1981). This study is a further step forward in the framework of an ongoing study of integrative taxonomy of this complex (Giacò et al. 2021).

## Materials and methods

### Collection of material

Material for karyological analyses was collected for all the 14 species belonging to the complex, for a total of 39 populations (Table S1). It includes material newly collected for this study and measurable metaphase plates already published in previous studies (Marchi and D'Amato 1973; Marchi et al. 1979; Valdés-Bermejo and Antúnez 1981). Fieldwork for seed collection was conducted from June to September 2020 and 2021 on 21 populations. For *S. chamaecyparissus*, given the unavailability of viable seeds in the sampled population, we prepared stem cuttings from two different individuals. The cuttings were planted in the Botanic Garden of Pisa, and root tips for karyological analyses were collected in winter. Seeds for six Sardinian populations were already available at Cagliari's seed bank (Banca del Germoplasma della Sardegna, BG-SAR).

### Karyological analyses

Metaphase plates were obtained by squashing root-tip cells according to the following protocol: pre-treatment in 0.4% colchicine solution for 3 hours; 3:1 ethanol/acetic-acid fixing solution for one hour; hydrolysis in HCl 1 N for seven to eight minutes at 60 °C; staining with leucobasic fuchsine for at least two hours. Metaphase plates were observed with a Leitz Diaplan microscope, were photographed with a Leica MC170 HD camera and related software (LAS EZ 3.4.0), and were measured using the software KaryoType (Altınordu et al. 2016). Images of metaphase plates that were already available in literature (Marchi and D'Amato 1973; Marchi et al. 1979; Valdés-Bermejo and Antúnez 1981) were digitized and measured using the same software. As regards the populations sampled in the field and those coming from BG-SAR, four to seven metaphase plates from different individuals for each population were measured.

THL (Total Haploid Length, i.e. the length of the monoploid chromosome complement) and the indices $CV_{CL}$ and $M_{CA}$ were calculated for each metaphase plate. $CV_{CL}$ (Coefficient of Variation of Chromosome Length) quantifies the inter-chromosomal asymmetry, and it is calculated by the ratio between standard deviation and mean of chromosome length × 100 (Paszko 2006). $M_{CA}$ (Mean Centromeric Asymmetry) quantifies the intra-chromosomal asymmetry, and it is calculated as the mean of $(L-S)/(L+S) \times 100$, where "L" stands for long arm and "S" stands for short arm of each chromosome in the complement (Peruzzi and Eroğlu 2013). Chromosomes are classified based on Levan's nomenclature (Levan et al. 1964). Differences among ploidy levels were investigated with Kruskal-Wallis test followed by pairwise Mann-Whitney U-Test with Bonferroni correction. Differences were considered significant when $p < 0.01$. Statistical analyses were conducted using R software (R: A language and environment for statistical URL: https://www.R-project.org/.).

Idiograms presented in Figure 1 were obtained based on the average measures reported in Tables S2 and S3, respectively. Chromosomes of different metaphase plates were grouped following the graphical method proposed by Plummer et al. (2003). In this method, New Relative Length (100 × chromosome length of the single chromosome/total length of all chromosomes) is plotted against arm ratio. In this representation, putatively homologous chromosomes are displayed close each other.

## Results

### Ploidy levels

Five different ploidy levels were detected: diploid, triploid, tetraploid, pentaploid, and hexaploid. The triploid level is referred to a single individual detected in the studied population of *S. virens*, that is otherwise diploid. Chromosome number is provided for the first time for *S. benthamiana* and *S. vedranensis*, that are both diploid. A new hexaploid cytotype was detected for *S. villosa* in southern Spain. A summary of ploidy levels and mean values of THL, $CV_{CL}$, and $M_{CA}$ is reported in Table 1.

### Karyomorphology and karyotype asymmetry

The two studied populations of *S. benthamiana* are diploid ($2n = 2x = 18$ chromosomes) and show 8 pairs of metacentric (*m*)/submetacentric (*sm*) chromosomes and one pair of subtelocentric (*st*) chromosomes (Figures 1 and 2A, Table S2). The longest chromosome of the complement is 6.3 µm ±

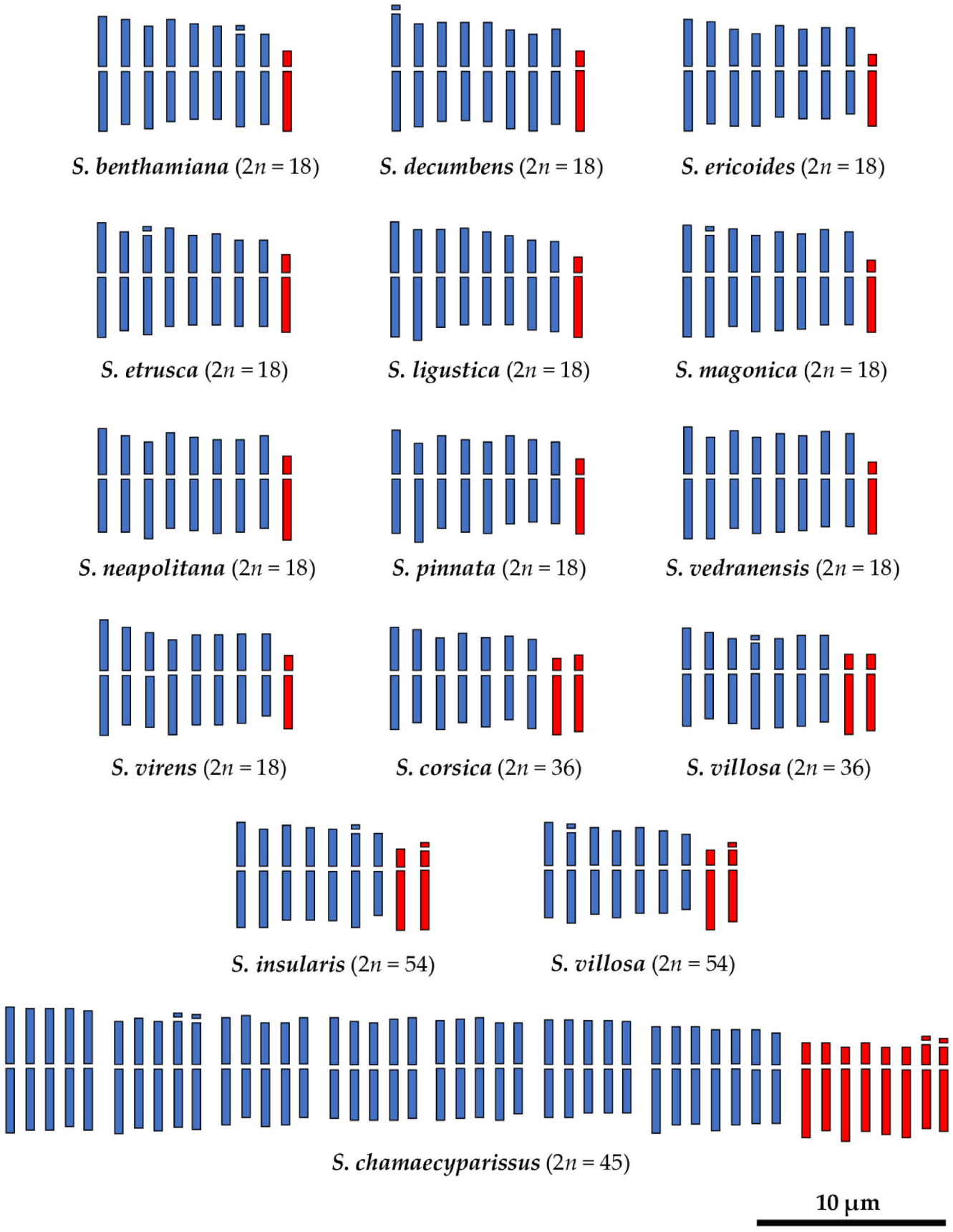


**Figure 1.** Monoploid idiograms ($x = 9$) of the studied species of the *Santolina chamaecyparissus* complex, based on the mean values reported in Table S2. For *Santolina chamaecyparissus* a holoploid idiogram is presented, based on the mean values reported in Table S3. The last 15 chromosomes of *S. chamaecyparissus* cannot be grouped by five on morphological grounds. Consequently, according to their mean arm ratio, they have been grouped in one group of seven submetacentric chromosomes, and one group of eight subtelocentric chromosomes. Metacentric and submetacentric chromosomes are coloured in blue, while subtelocentric chromosomes are coloured in red.

**Table 1.** Karyological data in the *Santolina chamaecyparissus* complex.

| Species | 2*n* | THL (µm) | $CV_{CL}$ | $M_{CA}$ |
|---|---|---|---|---|
| *S. benthamiana* Jord. & Fourr. | 18 | 49.1 (± 6.4) | 13.8 (± 2.1) | 18.2 (± 1.8) |
| *S. chamaecyparissus* L. | 45 | 43.9 (± 7.1) | 16.8 (± 1.7) | 20.9 (± 1.7) |
| *S. corsica* Jord. & Fourr. | 36 | 41.5 (± 10.2) | 13.2 (± 4.0) | 25.9 (± 1.5) |
| *S. decumbens* Mill. | 18 | 44.1 (± 8.1) | 13.1 (± 2.7) | 17.7 (± 2.0) |
| *S. ericoides* Poir. | 18 | 43.6 (± 6.3) | 14.5 (± 2.9) | 17.8 (± 1.9) |
| *S. etrusca* (Lacaita) Marchi & D'Amato | 18 | 50.5 (± 11.5) | 13.9 (± 2.3) | 16.8 (± 2.2) |
| *S. insularis* (Gennari ex Fiori) Arrigoni | 54 | 45.9 (± 8.1) | 14.1 (± 2.6) | 24.1 (± 2.0) |
| *S. ligustica* Arrigoni | 18 | 40.1 (± 4.7) | 14.1 (± 2.1) | 18.7 (± 1.2) |
| *S. magonica* (O.Bolòs, Molin. & P.Monts.) Romo | 18 | 48.9 (± 13.6) | 13.3 (± 3.1) | 16.6 (± 1.7) |
| *S. neapolitana* Jord. & Fourr. | 18 | 47.5 (± 4.4) | 11.9 (± 3.0) | 18.0 (± 1.6) |
| *S. pinnata* Viv. | 18 | 49.5 (± 7.3) | 11.1 (± 1.4) | 18.6 (± 1.4) |
| *S. vedranensis* (O.Bolòs & Vigo) L.Sáez M.Serrano, S.Ortiz & R.Carbajal | 18 | 54.6 (± 6.5) | 13.6 (± 2.6) | 17.5 (± 1.5) |
| *S. villosa* Mill. | 36 | 41.5 (± 5.8) | 12.3 (± 2.9) | 24.7 (± 2.5) |
| | 54 | 45.1 (± 6.6) | 14.2 (± 2.4) | 27.0 (± 1.5) |
| *S. virens* Mill. | 18 | 42.2 (± 5.4) | 15.9 (± 2.9) | 18.8 (± 2.1) |

THL = Total Haploid (monoploid) Length; $CV_{CL}$ = Coefficient of Variation of Chromosome Length; $M_{CA}$ = Mean Centromeric Asymmetry. Values of THL, $CV_{CL}$, and $M_{CA}$ are reported as mean (± standard deviation).

1.0 µm long on average, and the shortest chromosome is 4.3 µm ± 0.8 µm long on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. Secondary constrictions were occasionally observed in the population from Le Roumenga (Occitanie, France) on a *m* chromosome pair and on a *sm* chromosome pair (Figure S1A–C).

The studied naturalized population of *S. chamaecyparissus* is pentaploid (2*n* = 5*x* = 45 chromosomes), and it shows 37 *m*/*sm* chromosomes and 8 *st* chromosomes (Figures 1 and 2B, Table S3). The karyotype of this species is irregular since not all chromosomes can be grouped by five on morphological grounds (Figure 1). The longest chromosome of the complement is 6.5 µm ± 1.0 µm long on average, and the shortest chromosome is 4.3 µm ± 1.0 µm long on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. Secondary constrictions were observed on three *sm* chromosomes and on a single *st* chromosome (Figure 2B).

The two studied populations of *S. corsica* are tetraploid (2*n* = 4*x* = 36 chromosomes) and chromosomes can be easily grouped by four on morphological grounds. This species shows 7 groups of four *m*/*sm* chromosomes and 2 groups of four *st* chromosomes (Figures 1 and 2C, Table S2). THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. The longest chromosome of the complement is 5.7 µm ± 1.8 µm long on average, and the shortest chromosome is 4.1 µm ± 0.9 µm long on average. Secondary constrictions were occasionally observed in both populations on *sm* and *st* chromosome pairs (Figure S1D–F).

The three studied populations of *S. decumbens* are diploid (2*n* = 2*x* = 18 chromosomes) and show 8 pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2D, Table S2). The longest chromosome of the complement is 6.0 µm ± 1.0 µm long on average, and the shortest chromosome is 4.0 µm ± 0.7 µm long on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. Secondary constrictions were observed in a single metaphase plate (La Fare-les-Oliviers, Provence-Alpes-Côte d'Azur, France) on a pair of *m* chromosomes (Figure S1G).

The three studied populations of *S. ericoides* are diploid (2*n* = 2*x* = 18 chromosomes). This species shows 8 pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2E, Table S2). The longest chromosome of the complement is 5.6 µm ± 0.9 µm long on average, and the shortest chromosome is 3.7 µm ± 0.6 µm long on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. Secondary constrictions were occasionally observed on *sm* and *st* chromosome pairs (Figure S1H–J).

The two studied populations of *S. etrusca* are diploid (2*n* = 2*x* = 18 chromosomes) and show 8 pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2F, Table S2). The longest chromosome of the complement is 6.8 µm ± 2.0 µm long on average, and the shortest chromosome is 4.5 µm ± 1.0 µm long on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. Secondary constrictions were observed in a single metaphase plate (Radicofani, Tuscany, Italy) on a *m* chromosome and on a pair of *sm* chromosomes (Figure S1K).

The five studied populations of *S. insularis* are hexaploid (2*n* = 6*x* = 54 chromosomes). The chromosomes of this species can be easily grouped by six on morphological grounds, indeed this species shows 7 groups of *m*/*sm* chromosomes and 2 groups of *st* chromosomes (Figures 1 and 2G, Table S2). The longest chromosome of the complement is 6.1 µm ± 1.4 µm long on average, and the shortest chromosome is 4.8 µm ± 1.0 µm long on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. Secondary constrictions were occasionally observed on *sm* and *st* chromosome pairs (Figure S1L–N).

The studied population of *S. ligustica* is diploid (2*n* = 2*x* = 18 chromosomes) and shows eight pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2H, Table S2). The longest chromosome of the complement is 5.5 µm ± 0.6 µm on average, and the shortest chromosome is 3.8 µm ± 0.4 µm on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. We did not observe secondary constrictions in this species.

The two studied populations of *S. magonica* are diploid (2*n* = 2*x* = 18 chromosomes) and show eight pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2I, Table S2). The longest chromosome of the complement is 6.5 µm ± 1.9 µm on average, and the shortest chromosome is 4.6 µm ± 1.5 µm on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. Secondary constrictions were detected in two metaphase plates of the population from Tirant (Menorca, Balearic Islands) on a pair of *sm* chromosomes (Figure 3I and Figure S1O).

The studied population of *S. neapolitana* is diploid ($2n = 2x = 18$ chromosomes) and shows eight pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2J, Table S2). The longest chromosome of the complement is 6.2 µm ± 1.0 on average, and the shortest chromosome is 4.6 µm ± 0.5 µm on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. We did not observe secondary constrictions in this species.

The studied population of *S. pinnata* is diploid ($2n = 2x = 18$ chromosomes) and shows eight pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2K, Table S2). The longest chromosome of the complement is 6.1 µm ± 1.0 on average, and the shortest chromosome is 4.4 µm ± 0.6 µm on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. We did not observe secondary constrictions in this species.

The studied population of *S. vedranensis* is diploid ($2n = 2x = 18$ chromosomes) and shows eight pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2L, Table S2). The longest chromosome of the

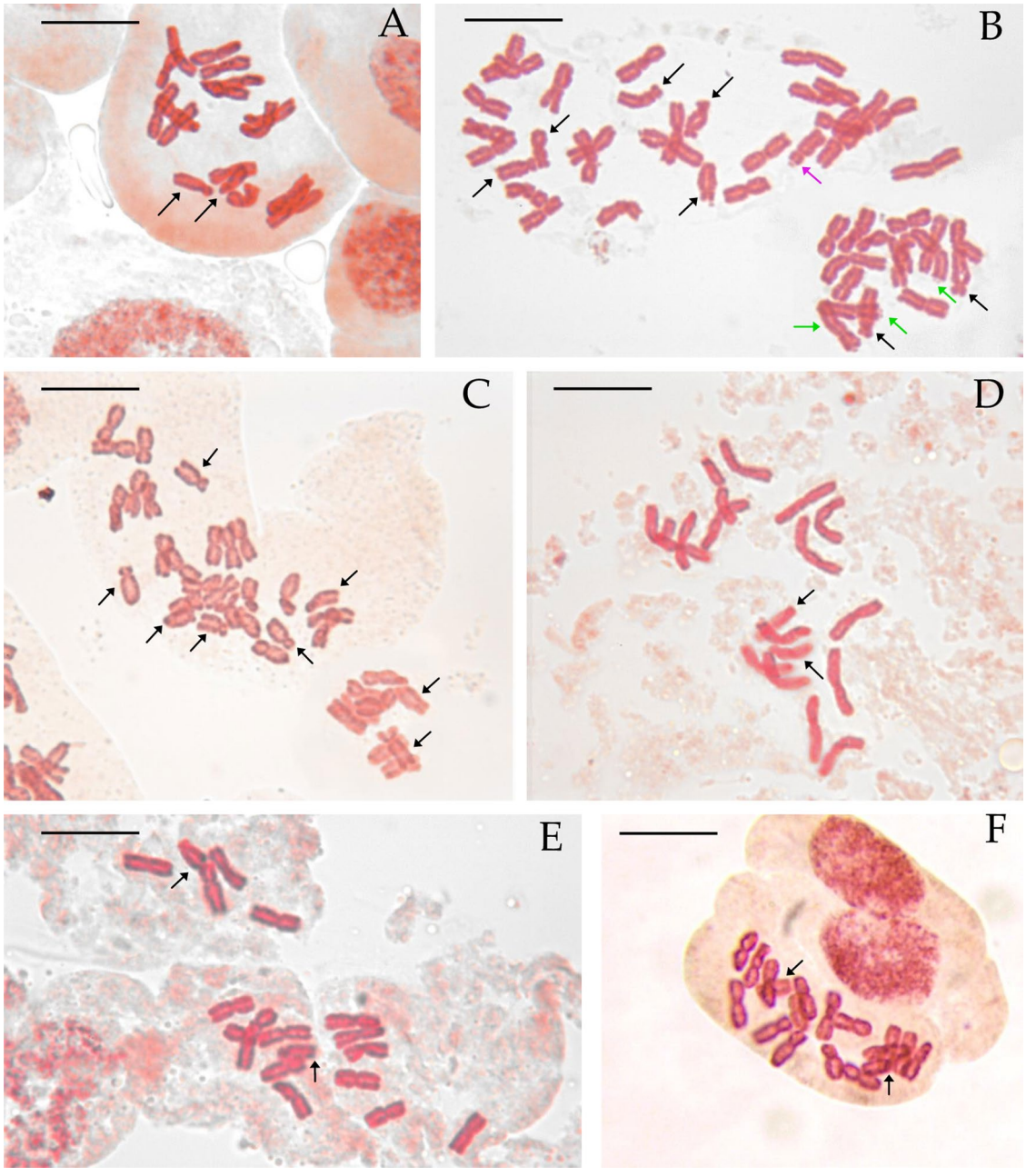


**Figure 2.** Selected metaphase plates in species of the *Santolina chamaecyparissus* complex. *Santolina benthamiana* (A, $2n = 2x = 18$, Prats-de-Mollo-la-Preste, Occitanie, France), *S. chamaecyparissus* (B, $2n = 5x = 45$, Le Luc, Provence-Alpes-Côte d'Azur, France), *S. corsica* (C, $2n = 4x = 36$, Mont Pigno, Corsica, France), *S. decumbens* (D, $2n = 2x = 18$, Mont Caume, Provence-Alpes-Côte d'Azur, France), *S. ericoides* (E, $2n = 2x = 18$, Béziers, Occitanie, France), *S. etrusca* (F, $2n = 2x = 18$, Radicofani, Tuscany, Italy), *S. insularis* (G, $2n = 6x = 54$, Iglesias, Sardinia, Italy), *S. ligustica* (H, $2n = 2x = 18$, Levanto, Liguria Italy), *S. magonica* (I, $2n = 2x = 18$, Tirant [Menorca], Balearic Islands, Spain), *S. neapolitana* (J, $2n = 2x = 18$, Monte Sant'Angelo, Campania, Italy), *S. pinnata* (K, $2n = 2x = 18$, Forno [Apuan Alps], Tuscany, Italy), *S. vedranensis* (L, $2n = 2x = 18$, Es Vedrà, Balearic Islands, Spain), *S. villosa* (M, $2n = 6x = 54$, Gor, Granada, Spain), *S. villosa* (N, $2n = 4x = 36$, Arganda, Madrid, Spain), *S. virens* (O, $2n = 2x = 18$, Fuentenebro, Castile and León, Spain). Individual with an additional metacentric chromosome (P) and a triploid individual (Q, $2n = 3x = 27$) of the same population of *S. virens*. Black arrows point at *st* chromosomes, green arrows point at *m* and *sm* chromosomes showing secondary constrictions, and purple arrows point at *st* chromosomes showing secondary constrictions. Scale bar = 10 µm.

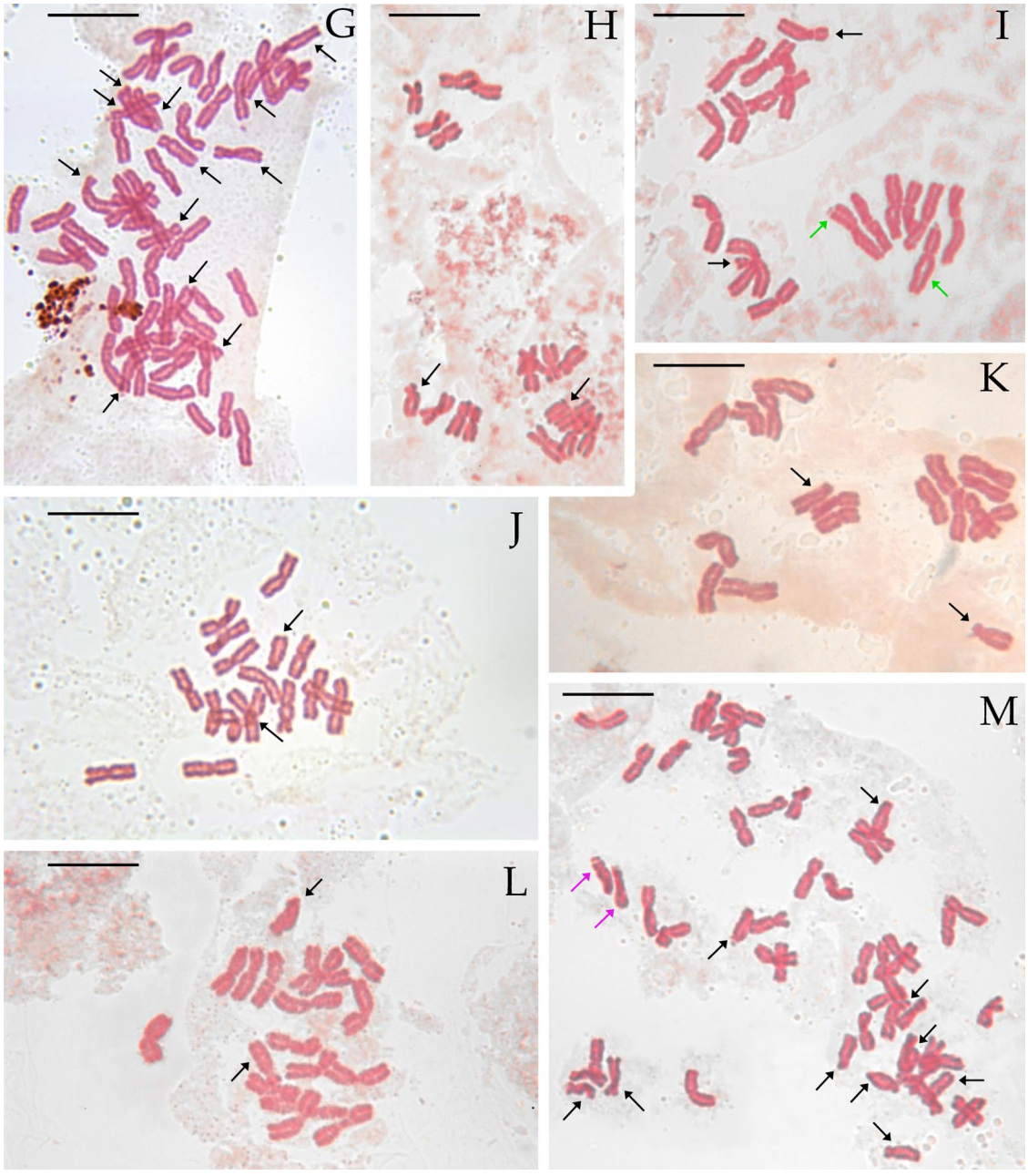


**Figure 2.** Continued.

complement is 7.4 µm ± 1.3 on average, and the shortest chromosome is 4.8 µm ± 0.6 µm on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. We did not observe secondary constrictions in this species.

The karyotype structure of the two cytotypes of *S. villosa* is very similar. The chromosomes of this species can be easily grouped by four in tetraploids and by six in hexaploids. In both cases, the monoploid set shows seven *m*/*sm* chromosomes and two *st* chromosomes (Figures 1 and 2M, 2N, Table S2). As regards the tetraploid cytotype, the longest chromosome of the complement is 5.9 µm ± 1.1 µm on average, and the shortest chromosome is 4.5 µm ± 0.6 µm on average. In the studied hexaploid population, the longest chromosome of the complement is 5.9 µm ± 1.4 µm on average, and the shortest chromosome is 4.6 µm ± 0.9 µm on average. Secondary constrictions were occasionally observed on *sm* and *st* chromosome pairs (Figure 2N, 2M, and 2P). THL, $CV_{CL}$, and $M_{CA}$ mean values for both cytotypes are reported in Table 1. Mann-Whitney U-Test suggests that there is no significant difference in THL (statistic = 19.5, $p = 0.34$), $CV_{CL}$ (statistic = 20, $p = 0.32$), and $M_{CA}$ (statistic = 26, $p = 0.03$) between the two cytotypes of *S. villosa*.

The studied population of *S. virens* shows mostly diploid ($2n = 2x = 18$ chromosomes) individuals, with a karyotype made by eight pairs of *m*/*sm* chromosomes and one pair of *st* chromosomes (Figures 1 and 2O, Table S2). The longest chromosome of the complement is 5.8 µm ± 0.8 on average, and the shortest chromosome is 3.5 µm ± 0.7 µm on average. THL, $CV_{CL}$, and $M_{CA}$ mean values are reported in Table 1. We detected a single individual with an additional metacentric

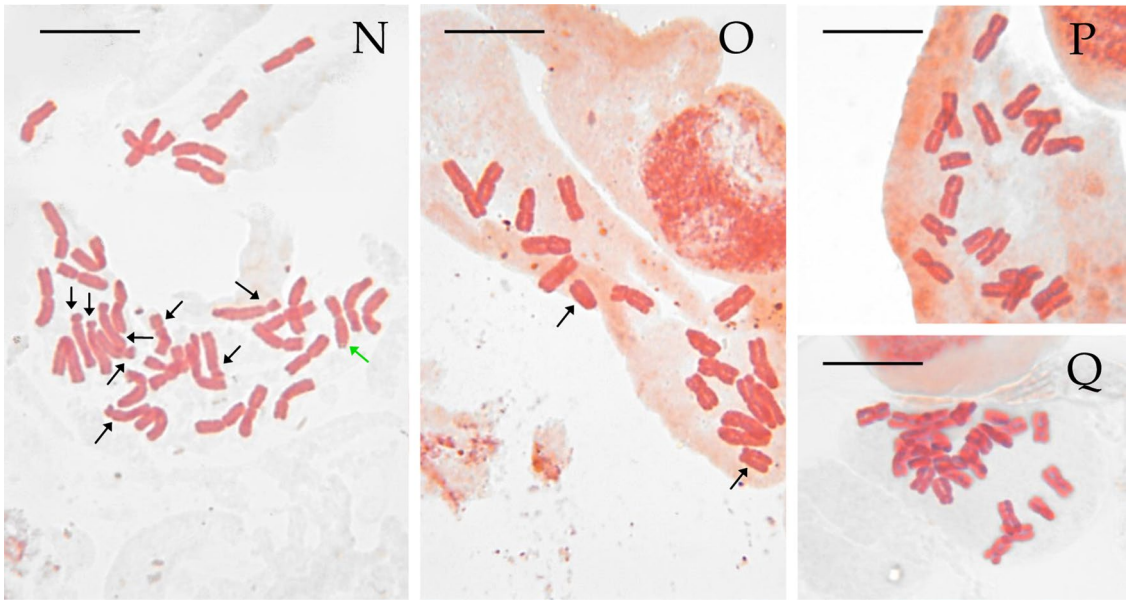


**Figure 2.** Continued.

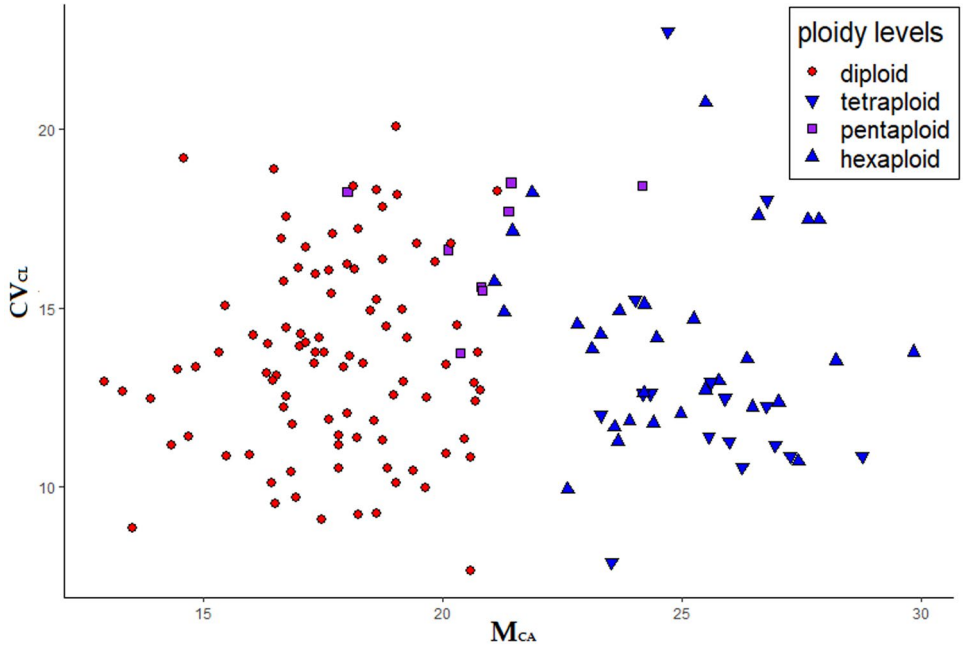


**Figure 3.** Karyotype asymmetry in the *Santolina chamaecyparissus* complex. The scatter plot shows the karyotype asymmetry of each measured metaphase plate (see also Table S1), expressed by Mean Centromeric Asymmetry (on x-axis) and Coefficient of Variation of Chromosome Length (on y-axis) values.

chromosome ($2n = 19$; Figure 2P) and a single triploid individual with $2n = 3x = 27$ chromosomes (Figure 2Q). Unfortunately, the three metaphase plates obtained from the triploid individual were good enough for counting chromosomes but not for karyotype reconstruction. We did not observe secondary constrictions in this species.

The scatter plot represented in Figure 3 suggests differences in $M_{CA}$ values among ploidy levels. Overall differences among the four ploidy levels are significant according to Kruskal-Wallis test (statistic = 100.4, $p$ 0.01). Pairwise Mann-Whitney U-Test with Bonferroni correction suggests that diploids show significantly ($p$ 0.01) lower $M_{CA}$ values with respect to tetraploids and hexaploids. *Santolina chamaecyparissus*, the only pentaploid species, shows significantly ($p < 0.01$) lower $M_{CA}$ values when compared to hexaploids, while there is no significant difference between *S. chamaecyparissus* and both diploids ($p = 0.01$, value on threshold) and tetraploids ($p = 0.02$). There is no significant difference in $M_{CA}$ values between tetraploids and hexaploids. Also, there is no significant difference among ploidy levels concerning $CV_{CL}$ and THL.

## Discussion

The chromosome numbers detected so far in the *S. chamaecyparissus* complex ($2n = 18$ [19], 27, 36 [37], 45, and 54) point

to a basic chromosome number $x = 9$. These findings confirm the previous studies concerning the genus *Santolina* (Marchi and D'Amato 1973; Marchi et al. 1979; Valdés-Bermejo and Antúnez 1981) and the subtribe Santolininae (Oberprieler et al. 2007); $x = 9$ is also the most likely ancestral chromosome number inferred for Asteraceae as a whole (Carta et al. 2020).

All the continental species except *S. villosa* are diploid, as also the two species endemic to the Balearic Islands, *S. magonica* (Mallorca and Menorca) and *S. vedranensis* (islet of Es Vedrà). Chromosome number was provided for the first time for *S. benthamiana* and *S. vedranensis*, that are both diploids. Moreover, a new hexaploid cytotype was found in *S. villosa*. For all the other species, previously published chromosome numbers are confirmed (see also the distribution map of the studied populations, Figure S2) (Marchi and D'Amato 1973; Arrigoni 1977; Marchi et al. 1979; Valdés-Bermejo and Antúnez 1981).

With Feulgen staining, secondary constrictions are not always visible. For this reason, we consider with caution the putative differences observed among species in this respect.

Diploid species show a rather homogeneous karyotype structure, despite some (not statistically tested) qualitative difference concerning the visibility/position of secondary constrictions. Polyploids also share a homogeneous karyotype structure, but different from that detected in diploids. Indeed, a significant difference in intra-chromosomal asymmetry ($M_{CA}$ values) is apparent between diploids and polyploids, except for the pentaploid *S. chamaecyparissus*, which shows a somehow intermediate karyotype structure (Figure 3). Indeed, considering the haploid (monoploid) chromosome set, all tetraploids and hexaploids show two subtelocentric chromosomes (*st*), while diploids only one. A similar difference was already documented in the two cytotypes (diploid and tetraploid) of *S. pectinata* Lag., a member of the *S. rosmarinifolia* L. complex, endemic to Spain (Rivero-Guerra 2008b). The different karyotype structure between diploids and polyploids in the *S. chamaecyparissus* complex may suggest an allopolyploid origin of the polyploids, involving also other diploid *Santolina* species, possibly extinct, likely having a different karyotype structure with respect to diploids studied here. However, autopolyploidy cannot be ruled out, since chromosome rearrangements may have occurred after polyploidization. Several studies demonstrated that translocations occur frequently in newly formed polyploids (Lim et al. 2008; Wang et al. 2012). These chromosomal changes are often involved in the diploidization process of polyploids (Dodsworth et al. 2016), to reduce sterility due to abnormal chromosome behavior during meiosis (Weiss and Maluszynska 2000; Levin 2002).

Irrespective of their allo- or autopolyploid origin, the karyotype structure of the polyploids *S. corsica* (tetraploid), *S. insularis* (hexaploid), and *S. villosa* (both tetraploid and hexaploid) is strikingly similar, since they show 7 metacentric/submetacentric chromosomes and 2 subtelocentric chromosomes in their monoploid complement (Figures 1 and 3). However, despite these polyploid species show a similar karyotype structure, preliminary molecular phylogenetic analyses (Varaldo et al. 2021) suggest that *S. corsica*/*S. insularis* (endemic to Corsica and Sardinia) and *S. villosa* (endemic to continental Spain) belong to two distinct clades. As regards this latter species, a tetraploid cytotype was already recorded in four different localities of central-eastern Spain by Valdés-Bermejo and Antúnez (1981), and a new hexaploid cytotype was recorded by us in southern Spain. There is no obvious morphological difference among these cytotypes, which both clearly agree with the descriptions provided by Carbajal et al. (2019) and Giacò et al. (2021). However, the lectotype of *S. villosa* lacks a precise geographical location (Ferrer-Gallego et al. 2021) so that, at the current state of knowledge, it is not possible to establish if this name applies to tetraploid or hexaploid plants. Accordingly, molecular and more detailed morphological studies are needed to clarify the possible taxonomic value of these cytotypes. Despite that, *S. villosa* was previously misidentified with *S. ericoides* (Carbajal et al. 2019), and both species are very common in central and eastern Spain (Figure S2). However, they show obvious differences on both morphological (Carbajal et al. 2019) and karyological grounds.

*Santolina insularis* is endemic to southern and central-eastern Sardinia, and all the studied populations are invariably hexaploid. On the contrary, *S. corsica*, which is endemic to Corsica and north-eastern Sardinia, is invariably tetraploid, fully confirming previous literature data (Marchi and D'Amato 1973; Marchi et al. 1979). The populations of these two species are allopatric in Sardinia, where *S. corsica* is reported only for Monte Albo (Angiolini and Bacchetta 2003). Although Arrigoni (Arrigoni 1982b) suggested that the two species may co-occur in central-eastern Sardinia, our chromosome counts do not support this hypothesis. Preliminary morphometric, seed morpho-colorimetric, and molecular analyses highlight a strong affinity between these two taxa, suggesting that *S. corsica* and *S. insularis* could even be considered as two cytotypes of the same species (A. Giacò and collaborators, in preparation).

*Santolina chamaecyparissus* is confirmed as pentaploid, and this explains the production of aborted pollen grains, as observed by Arrigoni (Arrigoni 1977). Its karyotype structure (Figure 1) and $M_{CA}$ values (Figure 3) are intermediate between the diploid and the other polyploid (artioploid) species. *Santolina chamaecyparissus* is of unknown, likely anthropic, origin and is commonly cultivated, albeit sometimes escapes from cultivation (Greuter 2008). According to our results, it is very likely an allopolyploid unit, and its morphology (Arrigoni 1977, 1982b) and karyotype suggest that at least one of the parental species is related to the lineage of *S. corsica*/*S. insularis*. However, the peculiar karyotype structure of *S. chamaecyparissus* cannot be explained by hypothesizing a simple crossing between any of the existing species in this complex.

*Santolina virens* is a commonly cultivated plant that is known as native only in Spain (Carbajal et al. 2019). According to Pau (1907) and Marcos and Burgaz (1990), *S. virens* may be a species with hybrid origin involving one species of the *S. chamaecyparissus* complex and a species of the *S. rosmarinifolia complex* as putative parental taxa. This hypothesis is strengthened by the karyotype instability suggested by the

documentation, in an otherwise diploid species (see also Kyohs, 1982), of a triploid individual and a diploid individual with an additional metacentric chromosome. In addition, in the same collection site of *S. virens* we also recorded the occurrence of *S. ericoides* and *S. rosmarinifolia*, that could represent the putative parental species. Future studies are needed to investigate the putative hybrid status of this species, also in relation with the two sympatric taxa mentioned above.

## Conclusion

Karyomorphological studies are still useful to investigate the diversity among species for systematic and taxonomic purposes. In this study, a complete picture of chromosome numbers and karyotype structure within the *S. chamaecyparissus* complex was presented, relying on 39 populations from all the 14 species of the complex (Table S1 and Figure S2). The main result of this study concerns the significant differences in the intra-chromosomal asymmetry ($M_{CA}$) between diploid and polyploid species (Figure 3). Indeed, all diploids invariably show only one subterminal chromosome while tetraploid and hexaploid species two. This feature suggests a possible allopolyploid origin of the polyploids, involving also other, possibly extinct, taxa. More evidence for an allopolyploid origin is provided by the peculiar karyotype structure of the pentaploid *S. chamaecyparissus*.

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