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Cytogenetics, biogeography and biology of *Santolina ageratifolia* Barnades ex Asso (Asteraceae: Anthemideae)

AIXA O. RIVERO-GUERRA*

Universidad de Sevilla. Departamento de Biología Vegetal y Ecología, Apdo 1095, 41080, Sevilla, Spain

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Santolina ageratifolia Barnades ex Asso is a natural autohexaploid ($2n = 6x = 54$, 54 + 1B), endemic to Teruel Province, Spain and inhabits a substrate derived from sandstone, red limolite and quartzite. Three chromosome formulae are found: 30m + 12sm + 12st, present in 52% of the descendants (52 metaphase), 24m + 5m-1sm + 18sm + 6st in 39% of the descendants (39 metaphase) and 24m + 5m-1sm + 18sm + 6st + 1B in 9% of the descendants (9 metaphase). Chiasmata are mostly interstitial rather than terminal, giving rise to the formation of cruciform structures. The predominance of bivalent configurations in the meiosis and the exclusively bivalent formation in four individuals indicate that this species has a strong tendency towards diploidization. Secondary association of bivalents is observed in the number of 2, 4–6 chromosomes associated, the average being 4.21 ± 1.20 chromosomes. The variation in the chromosomal characteristics suggests chromosome translocation and/or inversions. This species is partially sterile, with a mean pollen fertility of 40.56% and a mean fructification of 34.24%. The frequencies of multivalents and pollen fertility have a strongly significant effect on fructification percentage. Phenotypic variation in habits is not correlated with karyotype characteristics. The cytogeography of the polyploid taxa of the *Santolina rosmarinifolia* aggregate is discussed. *Botanical Journal of the Linnean Society*, 2008, **157**, 797–807.

ADDITIONAL KEYWORDS: diploidization – geological substrate – heteromorphic multivalents – multiple regression – nested MANOVA – pollen fertility – polyploidization – reproductive fitness – secondary association of bivalents – speciation.

INTRODUCTION

Polyploidy could represent a source of novel evolutionary process (Stebbins, 1971; Otto & Whitton, 2000). Polyploidization might be a source of genomic stress that facilitates rapid evolution. Genome restructuring and gene-level changes, including concerted evolution and gene silencing, also occurs in polyploids (Jackson, 1976; Soltis & Soltis, 1999). Polyploidization events, often by additional rounds of 'diploidization' and evolutionary divergence among previously doubled genomic sequences, also occur (Jackson, 1976; Soltis & Soltis, 1999; Wendel, 2000; Soltis, Soltis & Tate, 2003). Furthermore, gene silencing plays an important role in the regulation of duplicated genes (Jackson, 1976; Comai *et al.*, 2000; Levin, 2002). The diploidization process has been studied by Levan (1940) in *Allium porrum*, by Koul & Gohil (1970) in *Allium ampeloprasum*, by Sisodia (1970) in *Urochloa pullulans* and *Urochloa stolonifera*, by Díaz Lifante (1996) in *Asphodelus fistulosus* and *Asphodelus refreactus*, by Gatt *et al.* (1998) in allotetraploid species of *Dahlia*, by Sanderson *et al.* (1999) in *Sarcobatus*, by Ehrendorfer & Lambrou (2000) in *Takhtajania perrieri*, by Saitoh (2003) in *Cobitis striata*, by Ezaz, McAndrew & Penman (2004) in *Oreochromis niloticus*, etc.

The level of heterozygosity increased strongly with the ploidal level (Brochmann *et al.*, 2004). It is

*E-mail: rivero-guerra@hotmail.com

probable that the genetic variation stored as fixed heterozygosity within individual plants can be released via the occasional pairing of homologous chromosomes and by translocation events (Brochmann *et al.*, 2004). Although autopolyploids occur spontaneously in nature at relatively high rates, the likelihood of their establishment is low because of an initially high degree of sterility and the lack of immediate and stable novel gene combinations (Sang *et al.*, 2004).

It has long been known that the frequency of polyploidy increases with latitude in the northern hemisphere. Hagerup (1931) proposed that polyploids are better adapted than diploids to extreme climates. Stebbins (1971) argued that polyploids have greater ecological adaptability than do diploids. Ehrendorfer (1980) concluded that there are no direct general causal connections between polyploidy on the one hand and ecology, habitat or distribution on the other.

The magnitude of geographical differentiation is critical to understanding the maintenance of diploid and polyploid cytotypes within species and the role of polyploidization in the evolution of ecological tolerances. Geographical separation between polyploids and diploids can be maintained by either environmentally dependent or environmentally independent selection, as shown by Johnson, Husband & Burton (2003) among others. The geographical separation of taxa is often interpreted as the result of ecological sorting along an abiotic or biotic environmental gradient (Fowler & Levin, 1984).

The *Santolina rosmarinifolia* aggregate comprises eight taxa, principally diploids (five taxa). One of the taxa has two cytotypes – diploid and tetraploid – and the others are tetraploid and hexaploid. *Santolina rosmarinifolia* ssp. *rosmarinifolia* is located in the Central Iberian Peninsula, running northwards in the Peninsula, in the Occidental and Iberian Central Systems. The remaining species of the aggregate are located towards the periphery of its distribution.

The present paper is a continuation of a series intended to increase knowledge of the chromosomal variation of this aggregate and to clarify the taxonomic relationships of the species. *Santolina ageratifolia* is the only member of this group for which chromosome numbers have not been reported previously.

The objectives of the present study are to document for the first time the following aspects of *S. ageratifolia*: (1) the somatic chromosome number and interpopulation variation of chromosome morphology; (2) intra- and interpopulation variation of the meiotic configuration and of the frequency of chiasmata in diakinesis, as well as pollen fertility and reproductive fitness; and (3) the effect of multivalent frequency and percentage of pollen fertility on fructification percentage.

MATERIAL AND METHODS

Sampling

Twenty-six individuals of the Ródenas population (40°38′87″N–1°31′12″W, 1400 m) and 36 individuals of the population from the base of the Cerro San Ginés (San Ginés in the text) (40°38′06″N–1°29′19″W, 1430 m) were studied. Samples from both populations were collected in the summer of 1998, in Teruel Province, Spain.

Cytogenetics and pollen fertility

The study of somatic chromosomes was carried out on root-tip meristems obtained *in vitro* from germinating achenes collected from natural populations. The root tips were treated with 8-hydroxyquinoline (0.002 M) (Tjio & Levan, 1950) and fixed in Farmer's fluid (Löve & Löve, 1975). To study meiosis and pollen fertility, flower buds were fixed in Carnoy's fluid (Löve & Löve, 1975). The root tips and the anthers were stained according to Snow (1963) (alcoholic hydrochloric acid–carmine solution) and squashed individually on slides in 45% acetic acid.

Chromosome number and morphology (length of the short arm, length of the long arm, total length of the chromosome, excluding the satellite and chromosome ratio), the number of satellites and their position on the chromosomes, class and the chromosome formula (according to the terminology of Levan, Fredga & Sandberg, 1965), the chromosomal asymmetry indices (according to Romero Zarco, 1986) and the karyotype asymmetry (according to the classification of Stebbins, 1971) were established from the mitotic plates of nine and 11 individuals from San Ginés and Ródenas, respectively. In each individual, five metaphase plates with a similar degree of chromosome contraction were studied and their mean was calculated.

The study of meiotic configurations and chiasmata frequencies was carried out on 11 individuals per population. In each individual, three to five meiocytes were analysed. The meiotic configurations (univalent, bivalent and multivalent frequencies) were determined following the classification of Jackson & Casey (1982); therefore, the frequencies of terminal and proximal chiasmata and of interstitial chiasmata followed the classification of Sybenga (1975).

Pollen fertility was estimated by counting 300–400 mature pollen grains per plant, using cotton-blue stain. The total quantity of sterile pollen estimated was determined as the sum of the number of aborted pollen grains and the number of pollen grains not stained or tenuously stained. The pollen grains that showed the cytoplasm uniformly stained dark blue were considered viable.

Reproductive fitness

The number of flowers per capitulum and the number of achenes per capitulum were determined. For each character, three observations were carried out per individual. The fructification percentage was calculated as (number of achenes per capitulum × 100/ number of flowers per capitulum).

Statistical methods

Each individual (specimen) was treated as an independent operational taxonomic unit (OTU) for the whole statistical test, although dissimilarity among OTUs (individuals) and between groups of OTUs (populations) was also measured.

The MANOVA technique was applied to analyse the interpopulation variation of chromosome morphology (not including chromosome ratio). The nested MANOVA technique was applied to analyse the intra- and interpopulation variation of: (1) chromosome morphology in relation to the chromosome pairs (not including A1 and A2); (2) frequencies of meiotic configurations and of chiasmata; (3) pollen fertility; and (4) reproductive fitness. The variance components attributable to all the sources of variation analysed were calculated using the Variance Components.

The post-hoc test for chromosome morphology was carried out using the Bonferroni's method. A multiple regression technique was applied to evaluate the effect of the frequency of multivalents and pollen fertility on fructification percentage.

The techniques were applied after ensuring that requirements on data distribution were met: (1) multivariate normality by means of the Shapiro–Wilk contrast; (2) homogeneity of variance by means of the Barlett–Box contrast (Dytham, 2003; Grafen & Hails, 2003); (3) the presence of rare values or outliers, which were detected graphically, the MANOVA being especially sensitive to them; and (4) linearity of the observations and the error term (determined graphically) prior to multiple regression analysis. The characters (except for karyotype characters) were square-transformed prior to the analysis to increase the homogeneity of variance.

For statistical analysis, the statistical package STATISTICA, version 6.0, was used. The correlation coefficient was considered high when $r \geq 0.75$, moderate when $0.50 \leq r < 0.75$ and low when $r < 0.50$. Results were deemed significant if the probability of the null hypothesis was less than 0.05.

RESULTS

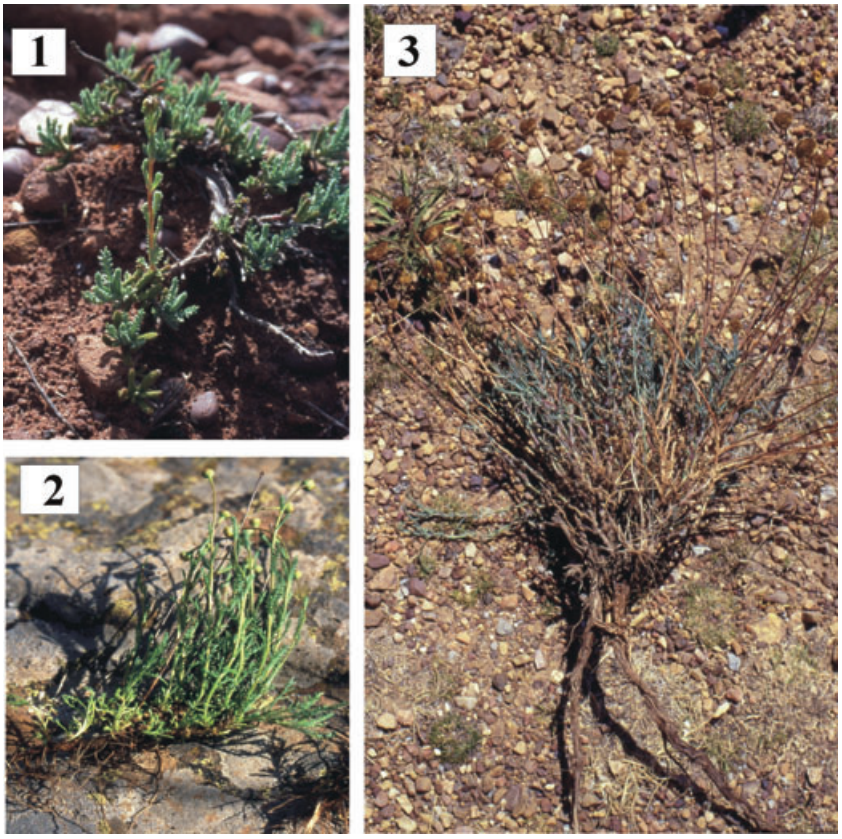
Geographical distribution and ecology of *Santolina ageratifolia*

Santolina ageratifolia is an endemic species of Teruel Province, Spain, of restricted geographical distribution. It is found at an altitude of 1370–1450 m, the average being 1396.00 ± 32.09 m, on two types of principally acid substrate. The Ródenas population is found on an inceptisol of Haploxerollic–Xerochrept type derived from a geological substrate of conglomerates, sandstone and red limolite of the Pliocene, while the population at the base of Cerro San Ginés is on a soil of Xerorthent–Xerumbrept type, a derivative of quartzite (Lower Ordovician) (Gabaldón, 1981a; J. López, pers. comm.). Individuals from the Ródenas population show decumbent rooting, thickly perennial woody basal stems (Figs 1, 2), in contrast to individuals from the San Ginés population, which are decumbent rooting and ascending (Fig. 3) with scarce lignification.

A voucher specimen (VAB 891262) gathered by Gonzalo Mateo on 10 June 1989 in Almohaja, Cerro del Ardal, has no reference to the geological substrate. Cerro del Ardal is found on quartzite and sandstone of the Upper Ordovician (Gabaldón, 1981a; López Udías & Fabregat Llueca, 2001). Another voucher specimen (MA 126816), gathered by Zapater in Griegos (Sierra de Albarracín), shows no record of either the collection date or the substrate it is taken from. In the search for this species in the location cited by Zapater, only *Santolina chamaecyparissus* was found. Griegos and the surroundings sit on a geological substrate of limestone oolites and pisolites, conglomerates, sandstone, clay and limestone of the Jurassic Period (Gabaldón, 1981b). In the zone, a small strip of sand, sandstone, gypsiferous marl and lacustrine limestone of the Lower Cretaceous is seen (Gabaldón, 1981b). These substrates do not correspond to the geological preferences of the species. A wide zone that extends over the whole Rio Griegos ravine consists of marl, grey limonite sandstone and sandstone of the Upper Jurassic Period (Gabaldón, 1981b); this voucher specimen may have been collected there. Furthermore, López Udías & Fabregat Llueca (2001) found three populations near to Ródenas (Morrón Blanco, Carravilla and Los Pozuelos) on acid substrate.

For the eight weather stations closest to the various study localities, meteorology data (rainfall and temperature) have been provided by the National Institute of Meteorology over a period of 20 years. The annual average temperature is 11.05 ± 6.60 °C. Rainfall is scarce the year round, the annual average being 360.96 ± 109.95 mm, guaranteeing aridity.

This species is sympatric with the diploid cytotypes of *S. chamaecyparissus* or coexists within the same populations. Vegetation in the zone is poor and consists of pastures and dwarf shrubs having a somewhat subnitrophilous character, together with *Biscutella atropurpurea*, *Filago minima*, *Peribalia involucrata*, *Pilosella castellana*, *Thymus izcoi, Cistus laurifolius, Calluna vulagaris, Linaria spartea,*



Figures 1–3. Habits of *Santolina ageratifolia*. Figs 1, 2. Ródenas, plant of decumbent-rooting habit on conglomerate and sandstone and red limolite. Fig. 3. Cerro San Ginés, plant of ascending habit on quartzite habitat.

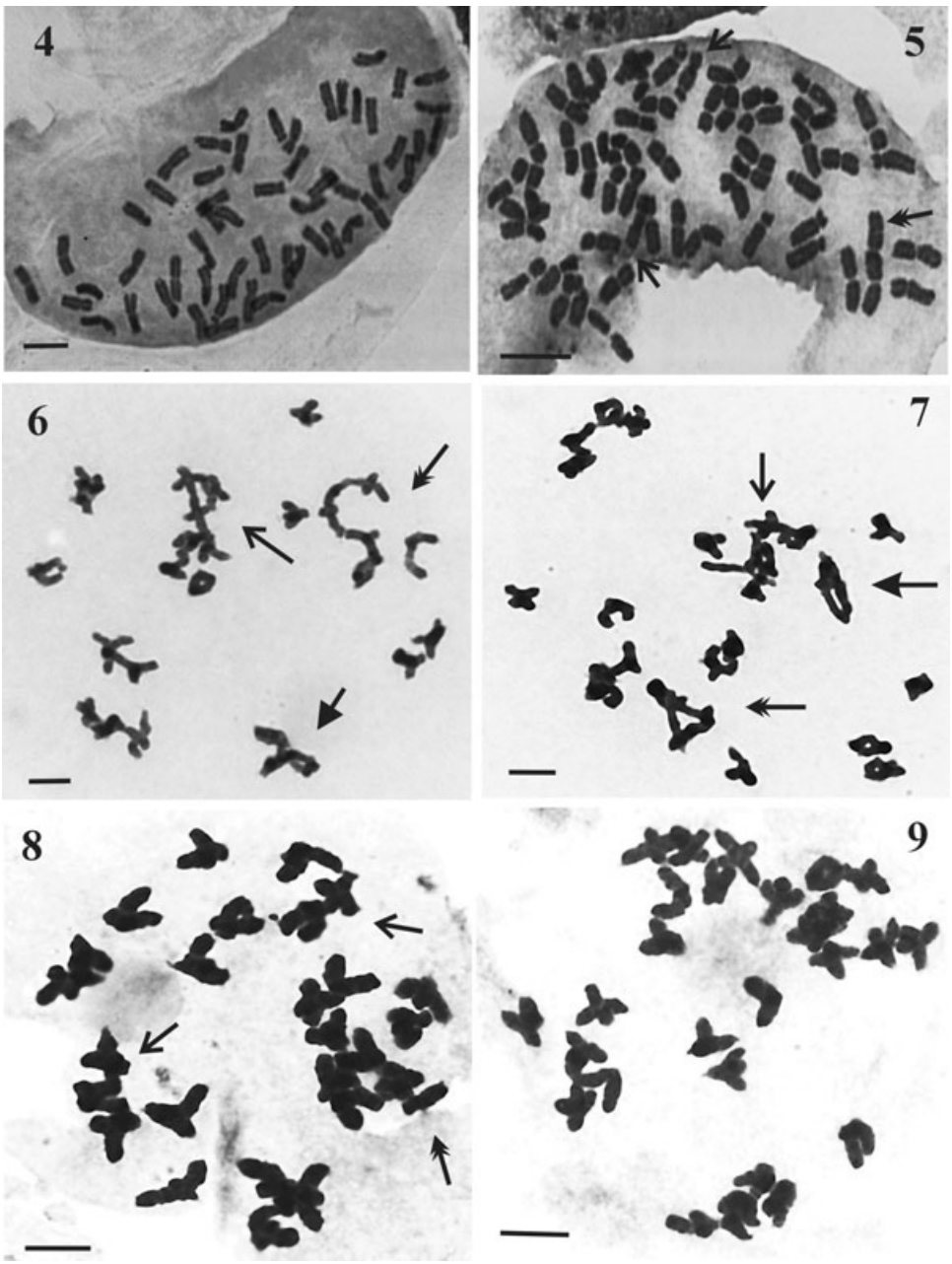
Stachys recta, Potentilla neumanniana, Plantago subulata, Aster aragonensis, Jasione crispa ssp. *sessiliflora, Koeleria vallesiana, Helianthemum asperum, Berberis garciae, Juniperus communis* and *Tuberaria guttata* (López Udías, Fabregat & Mateo, 1997; López Udías & Fabregat Llueca, 2001). In general, *S. ageratifolia* shows a preference for rocky, relatively arid, open habitats, with low vegetation and little biodiversity, where it encounters little competition. This confirms the general idea regarding the habitat of the endemic species of restricted geographical distribution referred to by Conti *et al.* (1999), Debussche & Thompson (2003), Lavergne *et al.* (2004, 2005) and Thompson *et al.* (2005).

Somatic chromosome number and chromosome morphology

Santolina ageratifolia is a hexaploid with a somatic chromosome number of $2n = 6x = 54$, 54 + 1B (Figs 4, 5). Three chromosome formulae, in accord with the classification of Levan *et al.* (1965), are found: 30m + 12sm + 12st, present in 52% of the descendants (52 metaphase) (Fig. 4); 24m + 5m – 1sm + 18sm + 6st in 39% of the descendants (39 metaphase); and 24m + 5m – 1sm + 18sm + 6st + 1B in 9% of the descendants (9 metaphase), with metacentric B chromosome (Fig. 5). Satellites are observed sporadically in metacentric and sub-metacentric chromosomes, a pair of sub-telocentric chromosomes with satellite being observed occasionally. The possibility of grouping the chromosomes into six suggests an autopolyploid origin for this species. Karyotype asymmetry is 2A.

The MANOVA analysis shows that karyotype characters are significantly different between populations (Wilk's $\lambda = 0.19$; $F_{5,15} = 12.75$; $P < 0.0001$) (Table 1). The variance components indicate that no chromosome character contributes strongly to population differentiation. The same analysis in relation to chromosome pairs shows statistical heterogeneity ($P < 0.0001$) between populations (Wilk's $\lambda = 0.33$; $F_{3,350} = 634.98$), between individuals in the populations (Wilk's $\lambda = 0.25$; $F_{54,2831.44} = 30.62$) and between chromosome pairs in each individual and population (Wilk's $\lambda = 0.15$; $F_{480,2850.73} = 5.26$).

The post-hoc test shows that the chromosome ratio, which defines each type of chromosome, shows



Figures 4–9. Karyotype and meiotic configurations in diakinesis of *Santolina ageratifolia*. Fig. 4. Somatic metaphase $2n = 6x = 54$ (Ródenas). Fig. 5. Somatic metaphase $2n = 6x = 54 + 1B$. The arrows indicate sub-metacentric chromosomes with secondary constriction in the long arm, the double arrow indicates metacentric B chromosome (Ródenas). Fig. 6. 1 OII + 11 CII + 1 CIV + 1 CVI + 1 CVIII + 1 CXII. The thick arrow indicates a hexavalent chain, the double arrow indicates the octavalent chain, the thin arrow indicates the heteromorphic dodecavalent chain (Ródenas). Fig. 7. 3 OII + 14 CII + 1 OIV + 1 CVI + 1 CX. The thick arrow indicates a ring of quadrivalents, the double arrow indicates a hexavalent chain and the thin arrow indicates a decavalent chain (Cerro San Ginés). Fig. 8. 1 I + 46 CII + 2 CIV. The arrows indicate a quadrivalent chain, the double arrow indicates a univalent (a metacentric B chromosome). Fig. 9. 1 OII + 26 CII. Scale bar, 6 μm.

Table 1. *Santolina ageratifolia* karyotype and its variability by means of MANOVA univariate analysis aimed at detecting variation between populations

Character	Range	Mean ± SD (μm)	CV (%)	Univariate analysis (dfe = 1; dfr = 19)	
				F (VCP) *P*	VCPR
LBC	0.58–2.57	1.66 ± 0.58	34.93	11.57 (50.70)†	49.30
LBL	2.36–3.41	3.06 ± 0.35	11.43	12.58 (53.00)†	47.00
LTC	3.90–5.96	4.72 ± 0.61	12.92	12.56 (52.90)†	47.10
A1	0.34–0.37	0.35 ± 0.03	8.57	5.37 (29.80)*	70.20
A2	0.09–0.12	0.10 ± 0.01	10.00	13.84 (55.50)†	44.50

A1, intrachromosomal asymmetry index; A2, interchromosomal asymmetry index; CV, coefficient of variation; LBC, length of short arm; LBL, length of long arm; LTC, total length of the chromosome; SD, standard deviation; VCP, variance components (%); VCPR, variance components in the error term.
*$P < 0.05$; †$P < 0.01$.

Table 2. Summary of meiotic configuration and chiasmata frequency of *Santolina ageratifolia* and their variability by means of nested MANOVA univariate analysis aimed at detecting variation within and between populations

Character	Range	Mean ± SD	CV (%)	APP (d.f. = 1)	AIP (d.f. = 20)	Error (d.f. = 45)
				F (VCP) *P*	*F* (VCP) *P*	VCP
BIV	1–27	12.86 ± 5.65	43.93	0.19 (0.45) NS	3.97 (49.40)†	50.15
MUL	0–7	4.03 ± 1.54	38.21	0.08 (0.10) NS	1.06 (2.10) NS	97.80
TOT	30–52	42.13 ± 4.22	10.02	0.03 (1.00) NS	1.54 (15.10) NS	83.90
TER	1–33	14.03 ± 6.62	47.18	18.48 (29.40)†	1.64 (12.30) NS	58.30
PRO	2–26	12.72 ± 5.97	46.93	4.84 (9.70)*	1.13 (3.70) NS	86.60
INT	12–41	27.97 ± 5.99	21.41	23.62 (36.30)†	1.49 (8.90) NS	54.80

AIP, between individuals in the populations; APP, between populations; BIV, total frequency of bivalents; CV, coefficient of variation; INT, total interstitial chiasmata frequency; MUL, total frequency of multivalents; PRO, proximal chiasmata frequency; SD, standard deviation; TER, terminal chiasmata frequency; TOT, total chiasmata frequency; VCP, variance components (%).
NS, not significant $P > 0.05$; *$P < 0.05$; †$P < 0.0001$.

significant differences ($P < 0.0001$) between subtelocentric and metacentric chromosome only (for the error term d.f. = 952; MS = 1.25). This indicates that the sub-metacentric chromosomes are not statistically distinguishable from the others.

Meiotic configuration and chiasmata frequency

Diakinesis is irregular (Figs 6–8). The average number of associations per cell [in (range)], from all the individuals studied, is 0.10 (0–1) univalents + 11.37 (1–26) rod bivalents + 1.49 (0–7) ring bivalents + 0.03 (0–2) ring trivalents + 1.63 (0–5) chain quadrivalents + 0.09 (0–1) ring quadrivalents + 0.01 (0–1) chain quinquevalents + 1.12 (0–3) chain hexavalents + 0.01 (0–1) ring hexavalents + 0.43 (0–1) chain octovalents + 0.33 (0–1) chain decavalents + 0.25 (0–1) chain dodecavalents (based on 67 cells in 22 individuals).

The study shows the predominance of bivalent configurations over multivalent configurations, where the rod bivalents predominate over ring bivalents. With regard to multivalent configurations, the quadrivalent and hexavalent chains are the most frequent, the octavalent chains being the most common configurations above the hexavalent level (Figs 6, 7). In addition, heteromorphic multivalents are observed (Fig. 6). The B chromosome is metacentric, slightly smaller than the smallest A chromosomes, with no differences in staining potential and no pairing with A chromosomes in the meiosis (Fig. 8). Chiasmata are mostly interstitial rather than terminal, giving rise to the formation of cruciform structures (Table 2, Figs 6–9). In general, this species shows a strong tendency towards diploidization. Secondary association of bivalents is observed in the number of 2, 4–6 chromosomes associated, the average being 4.21 ± 1.20 chromosomes (Figs 6–9). Four plants with

Table 3. Summary of the reproductive fitness of *Santolina ageratifolia*

Character	Range	Mean ± SD	CV (%)
Number of flowers per capitulum	58–285	122.37 ± 41.37	33.81
Achene number per capitulum	0–117	50.98 ± 19.01	37.28
Percentage of fructification (%)	0–97.78	34.24 ± 16.33	41.61

CV, coefficient of variation; SD, standard deviation.

exclusively bivalent formation are observed in Ródenas (Fig. 9).

The nested MANOVA shows significant differences between populations (Wilk's $\lambda = 0.60$; $F_{6,40} = 4.36$; $P < 0.01$) and between individuals in the populations (Wilk's $\lambda = 0.04$; $F_{120,238.63} = 1.48$; $P < 0.01$). Univariate analysis shows that total bivalent frequency and chiasmata frequency are the variables with significant differences at intra- and interpopulation level, respectively (Table 2).

Pollen fertility

The pollen is partially sterile. The average fertile pollen is 40.56 ± 25.67%, with a range of 8.00–88.00%. Pollen fertility shows statistical homogeneity ($P > 0.05$) between populations ($F_{1,45} = 0.07$) and between individuals in the populations ($F_{20,45} = 1.05$).

Reproductive fitness

All the characters show a wide range of variation (Table 3). Achene numbers per capitulum and percentage of fructification are low. These characters are not statistically significant ($P > 0.05$) for any of the variation sources analysed by means of nested MANOVA [between populations (Wilk's $\lambda = 0.99$; $F_{3,122} = 0.94$) and between individuals in the populations (Wilk's $\lambda = 0.34$; $F_{180,366.71} = 0.88$)]. Fructification percentage is strongly correlated statistically with pollen fertility and multivalent frequency (R^2 adjusted = 0.93; $F_{2,64} = 446.47$; $P < 0.0001$).

DISCUSSION

Cytogenetics

Santolina ageratifolia is a natural hexaploid with basic chromosome number $x = 9$, which agrees with that proposed for the genus *Santolina* by Valdés-Bermejo & Antúnez (1981) in the karyotype study of the Spanish species of this genus (not including *S. ageratifolia*) and for *S. pectinata* by Rivero-Guerra (2008).

The average lengths of the long and short arms, the average of chromosome length and the average of asymmetry indices for *S. ageratifolia* are lower than those found in the tetraploid cytotypes of *Santolina pectinata* (Rivero-Guerra, 2008). In other words, polyploidy produces a significant decrease of karyotype characteristics, in agreement with the results of Franklin de Melo *et al.* (1997) in Velloziaceae and of Solis Neffa & Fernández (2000) in *Turnera* (Turneraceae). Karyotype analysis also revealed a high frequency of m-type chromosomes, thus resulting in more symmetrical karyotypes. In general, karyotypes show low values of asymmetry, as is common in the tribe Anthemideae (Schweizer & Ehrendorfer, 1983).

The possibility of grouping the chromosomes into six and the large number of multivalents formed at meiosis suggest that this hexaploid is autopolyploid. However, the predominance of bivalent configurations in the meiosis and the exclusively bivalent formation in four individuals from Ródenas indicate that this species has a strong tendency towards diploidization.

Koul & Gohil (1970) and Kollmann (1972) considered that the absence of multivalents in *A. ampeloprasum* could be explained on the basis of the localization of the chiasmata in the centromeric region. In *S. ageratifolia* the chiasmata are attached mainly in the interstitial region, but no differences have been observed for chiasmata location per bivalent between individuals with multivalent chains and individuals with exclusively bivalent formation in the meiosis. Substitution of multivalents by bivalents in some individuals of *S. ageratifolia* boosts their fertility by eliminating all those abnormalities concomitant with polyploid meiosis. The complete restriction of homologous pairing is as a result of some form of genotypic control and bivalent may be the result of a multivalent suppressor system (Lacadena, 1996; Gatt *et al.*, 1998). In agreement with the contentions of Levan (1940), Koul & Gohil (1970) and Kollmann (1972), the localization of chiasmata in the centromeric region thus confers an advantage on polyploids by helping them in stabilizing and behaving as diploids. Levin (2002) suggests that the diploidization does not necessarily occur concurrently for all chromosomes or for all loci on a given chromosome. This might explain the absence of extensive diploidization for all the chromosome complement in some individuals of *S. ageratifolia* (Fig. 8).

Different authors have put forward different explanations for the causes of the secondary association of bivalents in the diakinesis and metaphase of the meiosis. For example, (1) Darlington & Moffett (1930), Lacadena & Puertas (1969) and Gupta & Roy (1973)

ascribed this phenomenon to the occurrence of residual attraction between bivalents composed of genetically or structurally similar pairs of chromosomes, analogous to the attraction which gives rise to primary prophase synapsis; (2) Gustafsson (1934) considered that it is a result of the affinity of chromosome pellicles; (3) Heilborn (1936) argued that it is a consequence of repulsion forces; (4) Thomas & Revell (1946) suggested that it is a result of the fusion of heterochromatic portions; (5) Jacob (1957) considered that it is a consequence of the homology between chromosomes; and (6) Riley (1960) and Kempanna & Riley (1964) in *Triticum aestivum* argued that these associations may be a relic of prophase attraction rather than secondary attractions of bivalents during diakinesis. The results of the present work suggest that theories 1, 5 and 6, above, may explain this phenomenon in *S. ageratifolia*.

Furthermore, the variation in the number of each type of chromosome and the presence of heteromorphic multivalents and of meiotic configurations above the hexavalent level, suggest that chromosome translocations and/or inversions occur, as in tetraploid cytotypes of *S. pectinata* (Rivero-Guerra, 2008). In addition, the B chromosome may arise by fusion of some chromosome fragments resulting from the translocation or inversion process.

Pollen fertility and reproductive fitness

This species is partially sterile and pollen fertility values are lower than those found by Khawaja, Ellis & Sybenga (1995) in the natural autohexaploid of *Lathyrus palustris* (56.2%), while fructification reached similar values (37%), but the range of variation is similar to that found by Atlagić (1996) in the hexaploid of *Helianthus annuus*. The pollen fertility and the percentage of fructification in this species are lower than those found in the tetraploid cytotypes of *S. rosmarinifolia* (87.38 and 57.10% respectively) and of *S. pectinata* (51.95 and 49.91% respectively) (A. O. Rivero-Guerra, unpubl. data). This indicates that the polyploidy has a negative effect on pollen fertility and on reproductive fitness in the polyploid taxa of the *S. rosmarinifolia* aggregate. Furthermore, *Santolina* plants are long-living, which would reduce the importance of high seed production.

Furthermore, multivalent frequency has a strongly significant effect on the fructification percentage. Usually, the orientation of the multivalent configurations is indifferent and presumably their segregations will be aberrant. This would lead to a moderate level of unbalanced gametes. The absence of aneuploid plants in this species suggests that these abnormal gametes (containing aneuploid chromosome numbers) do not participate in fertilization. Nevertheless, mature achenes are well formed and are viable, remaining so at 3.2 years (A. O. Rivero-Guerra, unpubl. data)

The characters of reproductive fitness are homogeneous for all the sources of variation analysed, indicating that the magnitude of their variation is maintained within and between populations. Phenotypic variations in habits observed between the two populations and between individuals in the San Ginés population are not correlated with cytogenetic characteristics; this may be a product of the genotype/environment interaction

Cytogeography of the polyploid taxa of the *S. rosmarinifolia* aggregate

Bowden (1940), Stebbins (1971) and Grant (1981), among others, have suggested that polyploidy is more common at higher latitudes and higher altitudes. The cytogeographical distribution patterns of the polyploids of this aggregate do not follow this general trend. For example, diploid cytotypes of *S. pectinata* occupy most of the species range, with tetraploid cytotypes clustered in the north-eastern portion (latitude 39°–40°N) (Rivero-Guerra, 2008). Similar patterns of parapatric distribution occur between the diploid and tetraploid cytotypes of *S. rosmarinifolia*, but the tetraploids are clustered in the western portion (Serra da Arrábida, latitude 38°N). Furthermore, *S. ageratifolia* is the only hexaploid of this aggregate with a restrictive geographical distribution in the eastern portion (latitude 40°N) at the border of the distribution of the aggregate. In general, polyploids of this aggregate have a disjunctive distribution, in 'islands' in the extreme west and in east of the Iberian Peninsula and a recent polyploidization process occurs in the centre and south (latitude 39°N) of the Iberian Peninsula. With regard to altitude, the polyploids inhabitat a lower altitude (mean 856.84 ± 316.43 m, $N = 26$ populations of polyploid taxa of the *S. rosmarinifolia* aggregate) than that of the diploids (mean 1128.21 ± 436.52 m, $N = 328$ populations of the diploid taxa of the *S. rosmarinifolia* aggregate). In general, diploid taxa characterize the entire range of the aggregate (latitude 36°–42°N) and show a broader ecological spectrum than that of polyploids.

The polyploid distribution patterns of the *S. rosmarinifolia* aggregate are in agreement with the contentions of Stebbins (1971), Levin (1983), Liu, Gituru & Wang (2004) and Rivero-Guerra (2008) about the ability of polyploids to colonize new areas and persist in habitats with different environmental conditions than their diploid precursors. Polyploids of this aggregate live under a lower temperature regimen (mean 11.30 ± 6.42 °C, $N = 13$ weather sta-

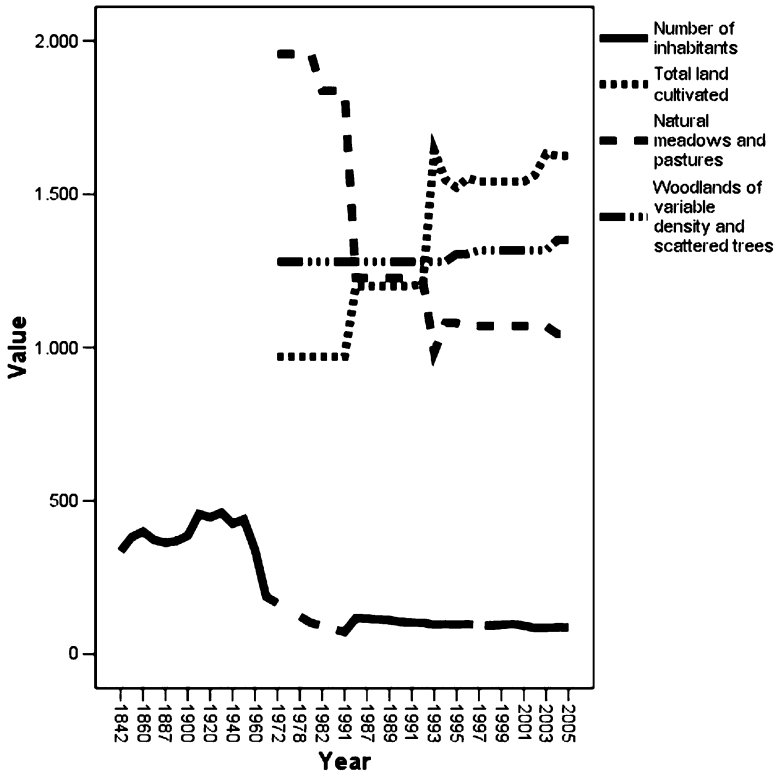


Figure 10. Rural population census and land use for the municipality of Ródenas.

tions) than that of diploids (mean 15.05 ± 8.97 °C, $N = 56$ weather stations) and under a lower regimen of annual rainfall (mean 377.33 ± 321.92 mm, $N = 26$ weather stations) than that of diploids (mean 648.99 ± 309.44 mm, $N = 250$ weather stations). This suggests that stressful ecological conditions (low temperature and low water availability) could have exerted selective pressure, resulting in chromosomal changes.

With regard to substrate preferences, polyploids have a less-diverse preference than do diploids (except for *S. impressa, S. semidentata* ssp. *melidensis*, *S. oblongifolia*, *S. viscosa* and *S. elegans*). For example, (1) diploid cytotypes of *S. pectinata* live on limestone, limestone and dolomites, marl–limestone, limestone and marl–limestone, limestone and marl, bioclastic limestone and conglomerate, limestone and sandstone and gypsiferous marl; whereas tetraploid cytotypes live on limestone, marl, gypsiferous marl and clay; (2) diploid cytotypes of *S. rosmarinifolia* live on various substrates: granites; conglomerates, sand, clay, sandstone, limestone and gypsum; marl and clay; marl–limestone; limestone and marl; slate, grauwackes, gravel, mud and clay; slate, sandstone, quartzite and limestone; whereas tetraploid cytotypes live on marl–limestone and sandstone and limestone conglomerate; however, (3) *S. ageratifolia*, the taxon with the highest ploidy level in this aggregate, lives on conglomerates and sandstone and red limolite and quartzite. This indicates that polyploidy may not have been a necessary precondition for adaptation to substrates.

The recent diversification process in these species and the disjunctive distribution of the polyploids arose from fragmentation or contraction of the species range, prevented gene flow between them and allowed fixation of the chromosomal changes, which favoured differentiation and, with this, allopatric speciation.

The karyotype characteristics, the geographical distributions and the morphological similarity with *S. pectinata* (leaf and involucral bract characters) (A. O. Rivero-Guerra, unpubl. data) suggest a close relationship between *S. pectinata* and *S. ageratifolia*. It is probably that this species derived from *S. pectinata*.

There are some major exciting questions remaining on *S. ageratifolia*: what are the physiological, morphological and evolutionary implications of polyploidy? Do these populations differ physiologically, genetically and morphologically?

CONSERVATION

López Udías & Fabregat Llueca (2001) estimated 54 146 plants in six populations of this species. The

probability of extinction for this species is extremely high as the populations are small and close to paths and roads in and around the village of Ródenas. It is highly vulnerable to human activity (agricultural and cattle), especially near Cerro San Ginés because of the agricultural use of the land in the surrounding area. At Ródenas, there has been a considerable decrease in the rural population since 1842 (Fig. 10) and much of the area then covered by natural meadows and pastures has been replaced by cultivated surface (dry wheat and barley fields) since 1978. The woodland area has suffered little alteration. All of this has drastic implications for the size of the *S. ageratifolia* populations (mainly that of San Ginés). This endemic species has persisted probably because of its capacity to grow in a habitat with scarce competitive interaction.

This work provides justification for the inclusion of this species as vulnerable in the *Red Book of Endangered Species* (IUCN Red List). Preservation of the genetic diversity of this species *in situ* should constitute a priority, as *S. ageratifolia* is an endangered species. The creation of a Natural Reserve in the zone would be ideal for its conservation.

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