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Phenotypic differentiation of peripheral populations of *Santolina rosmarinifolia* (Asteraceae)

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Phenotypic differentiation of two tetraploid ($2n = 4x = 36$, 36+1B, 36+2B) populations of *Santolina rosmarinifolia* geographically isolated from diploid populations was investigated. The karyotype was relatively homogeneous, meiosis was regular and pollen was fertile in both cytotypes. An autopolyploid or allopolyploid origin for tetraploid cytotypes is discussed. Overall, 80.82% of all variance in achene weight, time t_0, t_{50} and t_{90} of germination and accumulated germination rate was due to achene age at each ploidy level. Partition of the total phenotypic variance showed that there was extensive variation between ploidy levels. The mean of morphological characters was generally higher in polyploids. For diploid cytotypes, flower number, achene production and fruiting percentage were significantly higher than for tetraploid cytotypes. Cluster analysis indicated that the patterns of seedling morphology and development were similar in three diploid individuals and several tetraploids; the same analysis showed high similarity between diploid individuals of the natural populations, whereas tetraploid individuals showed high dissimilarity among themselves and with diploid individuals. Multiple correspondence analysis and logistic regression analysis indicated that qualitative characters contribute strongly to cytotype differentiation. The results support recognition of the tetraploid cytotypes at the subspecies level. *Botanical Journal of the Linnean Society*, 2008, **158**, 650–668.

ADDITIONAL KEYWORDS: cluster analysis – Iberian Peninsula – multidimensional scaling – nested multivariate analysis – pollen fertility – reproductive fitness – stepwise discriminant analysis.

INTRODUCTION

Darwin (1859) emphasized the effects of natural selection on species diversity and stated that this phenomenon operates most efficiently in large populations. However, he did not fully explain how species differed from locally adapted populations, how small isolated populations are able to evolve as a unit or the importance of gene flow, reproductive barriers and founder effects on speciation events. These issues were clarified by Mayr (1942, 1954) and Lewis (1953). Recent theoretical works suggest that peripheral populations (separated from central ones by spatial distance and/or by ecological divergence) are not especially adept at rapid evolution into a new niche (Rouhani & Barton, 1987; Charlesworth & Rouhani, 1988). Other theoretical models suggest rapid evolution for these populations and conclude that their geographical isolation arises from fragmentation or contraction of the species range, preventing gene flow between them. This is an important mechanism for allopatric speciation and for diversification of some plant groups and can cause the increase of phenotypic distance between peripheral and central populations (Levin, 1970; Lesica & Allendorf, 1995; García-Ramos & Kirkpatrick, 1997).

Peripherally isolated populations should show less genetic and phenotypic variation than central populations, as in *Sarracenia* L. (Schwaegerle & Schaal, 1979), *Betula* L. (Coyle, Sharik & Feret, 1982), *Gleditsia* L. (Schnabel & Hamrick, 1990), *Avena* L. (Jain, Rai & Singh, 1981) and *Oryza* L. (Thomas *et al.*, 2001), or show significant genetic and phenotypic differentiation, as in *Cyclamen* L. (Affre & Thompson, 1997).

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These populations are especially important for speciation events (Levin, 1970) because divergence between populations may also occur through the formation of novel polyploids. Although geographical isolation is not a prerequisite for speciation via polyploidy, ploidal shifts are local phenomena and one such shift is sufficient for species formation (Levin, 1993). The combination of the two processes may accelerate the speciation process in these populations as a result of stochastic processes and strong selection pressures. Although peripheral populations may be phenotypically similar to central populations (Lewis, 1966), they are usually reproductively isolated and constitute a unique evolutionary unit (Lesica & Allendorf, 1995).

The study of phenotypic differentiation between populations is a first step in determining the identity and relative importance of the evolutionary forces promoting or preventing differentiation (Armbruster, 1985; Domínguez *et al.*, 1998). Analysis of phenotypic variation of morphological characters can be useful in understanding how development of the pleiotropic process, disequilibrium linkage, the environment, genetic drift and natural selection may generate patterns of character variation within species (Armbruster, 1991; Endler, 1995).

Santolina rosmarinifolia L. (Asteraceae) is endemic to the Iberian Peninsula, growing in the central and northern parts on granitic substrates (López Udías, Fabregat & Mateo, 1997). It is normally diploid ($2n = 2x = 18$) (Valdés-Bermejo & Antúnez, 1981), but tetraploid cytotypes ($2n = 4x = 36$) were found in Serra da Arrábida, Portugal by Fernández & Queirós (1971). Those authors studied only one individual of the Azeitão population, which was hypotetraploid ($2n = 34$) and provided only a brief description of the karyotype of *S. rosmarinifolia*. In this species, no information is available regarding chromosome pairing at each ploidy, the mechanism to formation of polyploids in nature, seed germination, seedling development or partition of phenotypic variance.

This study attempted to test the prediction that geographically isolated tetraploid populations of *S. rosmarinifolia* show significant phenotypic differentiation from diploid populations of this species. The goals of this work were to: (1) quantify the cytological variation across the geographical range of *S. rosmarinifolia*; (2) examine meiotic configurations to determine the likely origin of *S. rosmarinifolia* polyploids; (3) determine how phenotypic variance is partitioned within and between ploidy levels; (4) determine whether diploid and tetraploid cytotypes exhibit subtle patterns of karyological, seed germination, seedling development and morphological differentiation.

MATERIAL AND METHODS

Sampling

Sixteen diploid populations (250 individuals in total) and two tetraploid populations (55 individuals in total) were studied. Diploid and tetraploid populations were collected in the summers of 1995 and 1997, respectively (Table 1).

Meteorology data

The meteorological data (rainfall and temperature) were provided by the National Institute of Meteorology of Spain and Portugal for the areas closest to the various study sites for a period of 20 years.

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). For the study of meiosis and pollen fertility, flower buds were fixed in the field 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 numbers and morphology [length of the short arm (LBC), length of the long arm (LBL), total length of the chromosome (LTC), excluding the satellite] and the chromosome ratio (length of the long arm/length of the short arm), 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 39 and 10 individuals from 13 diploid populations and two tetraploid populations, respectively (Table 1). 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 five diploid populations (151 meiocytes) and two tetraploid populations (54 meiocytes) (Table 1). 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); 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 was estimated as the sum of the number of aborted pollen

Table 1. Location of diploid and tetraploid cytotypes of *Santolina rosmarinifolia* (ROS). The number of individuals studied per population is shown

Pop.	Location	Ploidy levels	*N*				
			MT	MI	PO	PL	MM
1	Spain, Álava, Álava, Lantaron, Fontecha, 42°44′30′N 30°01′04′W, 464 m	2*x*	3	10	10	19	18
2	Spain, Ávila, Hoyos del Collado, 40°22′17′N 5°14′9′W, 1530 m	2*x*	3	–	–	–	13
3	Spain, Ávila, Hoyocasero, 40°21′54′N 4°11′49′W, 1300 m	2*x*	3	–	–	–	11
4	Spain, Ávila, Adanero, 40°53′32′N 4°37′17′W, 920 m	2*x*	–	10	10	19	15
5	Spain, Burgos, Belorado, 42°24′08′N 3°12′32′W, 793 m	2*x*	–	–	–	19	10
6	Spain, Madrid, El Escorial, 40°43′39′N 4°15′30′W, 1080 m	2*x*	–	–	–	–	11
7	Spain, Madrid, Sierra de Guadarrama, Miraflores de la Sierra, 40°48′21′N 3°46′37′W, 1150 m	2*x*	3	–	–	–	30
8	Spain, Madrid, Rascafría, 40°53′56′N 3°53′0.74′W, 1163 m	2*x*	3	–	–	–	15
9	Spain, Madrid, Valmayor reservoir, 40°31′18′N 4°2′55′W, 820 m	2*x*	3	–	–	–	13
10	Spain, Segovia, after coming out of Tunel de los Leones, 40°42′44′N 4°9′58′W, 1230 m	2*x*	3	10	10	–	14
11	Spain, Segovia, San Cristobal de la Vega, 41°6′39′N 4°38′11′W, 900 m	2*x*	3	–	–	20	11
12	Spain, Segovia, Cuéllar, 41°24′28′N 4°20′16′W, 870 m	2*x*	3	–	–	–	10
13	Spain, Segovia, Riofrio, 40°52′38′N 4°9′2.37′W, 1020 m	2*x*	3	10	10	–	32
14	Spain, Valladolid, Mojados, toward Megeces, 41°25′46′N 4°39′31′W, 720 m	2*x*	3	–	–	–	21
15	Spain, Valladolid, Olmedo, 41°18′25′N 4°41′0.8′W, 800 m	2*x*	3	–	–	19	13
16	Spain, Valladolid, Cogeces, 41°24′48′N 4°32′58′W, 720 m	2*x*	3	9	9	–	12
17	Portugal, Setúbal, Vila Nogueira de Azeitão, 38°29′55′N 9°01′03′W, 145 m	4*x*	5	9	9	30	38
18	Portugal, Sierra de Arrábida, in front of tile factory, 38°29′33′N 8°59′47′W, 160 m	4*x*	5	9	9	30	17

Pop, population; *N*, number of individuals studied; MT, mitosis; MI, meiosis; PO, pollen fertility; PL, morphology and development of the seedlings; MM, morphometric analysis of the natural populations.

grains and the number of pollen grains not stained or lightly stained. The pollen grains that showed the cytoplasm uniformly stained dark blue were considered viable.

Reproductive fitness

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

Achene germination

Diploid and tetraploid achenes were collected and stored at room temperature (approximately 20 °C). Germination was tested when the achenes were 0.2, 1.2, 2.2 and 3.2 years old. Each year, 100 achenes per population were set to germinate. Prior to sowing, the achenes were weighed and washed in a solution of 1% calcium hypochlorite. The achenes were sown in October on two layers of filter paper saturated with distilled water in sterilized 8 cm-diameter Petri dishes and sealed with Parafilm. Four replicates of 25 achenes per dish were used for each population. The dishes were put in a growth chamber with 16 h of fluorescent light (1200 lux intensity) at 15 °C and 8 h of darkness at 8 °C, a regime similar to natural conditions in the field during the study period. Germinated achenes were counted out daily for 39 days each year. An achene was considered to have germinated when the radicle emerged from the testa. Accumulated germination rate and the times t_0, t_{50} and t_{90} (number of days at which germination began and when 50% and 90% germination was reached, respectively) were noted. Achenes that did not germinate by the end of the study were subjected to a viability test using tetrazolium red.

Development and morphology of the seedlings

Seedlings were transplanted on the day of germination to flowerpots containing a mixture of soil and

perlite (3 : 1), supplied from the greenhouse of the Department of Plant Biology and Ecology at the University of Seville. Appendix 1 shows the quantitative characters studied in *S. rosmarinifolia.* The length of the cotyledons, length of the hypocotyl and length of the internode were measured when the appearance of the fifth leaf started. Seedling height was measured when the appearance of the twelfth leaf started. At the end of the study, the seedlings were pressed and dried in a drying room at 60 °C for two days and later weighed to determine the dry matter content per plant.

Morphometry of the natural populations

Quantitative and qualitative characters studied are shown in Appendix 1. They were selected according to their common use in *Santolina* taxonomy and variability observed between and within ploidy levels. The characters were evaluated in relation to the position of: (1) the leaves on flowering and sterile stems – basal, lower, middle, upper and fascicular (which arise from the axils of the cauline leaves of the sterile stems); (2) involucral bracts – outer, middle and the two well-defined inner rows – and interseminal bracts; (3) the flower and achene on the involucre – peripheral and central. For each character, three observations were made per individual, except for characters 14 and 15 (Appendix 1). Observations and measurements were performed under a binocular microscope and measurements were made with a digital calibrator. The terminology of Stearn (1996) was used.

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

Statistical methods

A principal component analysis (PCA) was employed to explore the correlation structure of the quantitative characters studied on meiosis, on morphology and development of the seedlings and on the natural populations to assess the relative importance of the characters to cytotype differentiation.

The nested MANOVA technique was applied to analyse variation in and between ploidy levels of chromosome morphology, reproductive fitness of natural populations, the accumulated germination rate and times t_0, t_{50} and t_{90} between ploidy levels, between achene age nested in each ploidy level and between populations nested in each ploidy level and achene age. The nested ANOVA technique was employed to analyse variation in: (1) PCA factors of meiotic characters (only within ploidy levels); (2) pollen fertility within and between ploidy levels; (3) PCA factors of the morphology and development of the seedlings; (4) PCA factors and dimensions of the quantitative and qualitative characters, respectively, of the natural populations. The post-hoc tests for germination variables and for chromosome morphology were carried out using the Bonferroni method.

Multidimensional scaling was employed to determine the correlation between qualitative characters of the natural populations and to determine which characters contribute most to dissimilarity between cytotypes. The contribution of these characters to cytotype differentiation was established by means of logistic regression technique and a Bonferroni correction was applied.

Stepwise discriminant analyses were performed individually for morphological and developmental characters of the seedlings and for quantitative characters of the natural populations to determine: (1) the group each individual belonged to with the highest probability; (2) the importance of each character for cytotype differentiation. Finally, morphological differentiation between diploid and tetraploid cytotypes was explored by means of cluster analysis, using an unweighted pair-group method with arithmetic averaging (UPGMA; Sokal & Michener, 1958) and Euclidean distances as the criterion for clustering.

The techniques were applied after ensuring that requirements on data distribution were met: (1) multivariate (MANOVA) or univariate (ANOVA) normality by means of the Shapiro–Wilk contrast; (2) homogeneity of variance by means of the Bartlett-Box contrast in the multivariate models and the Levene test in the univariate models (Grafen & Hails, 2003; Dytham, 2003); (3) the presence of rare values or outliers, which were detected graphically, MANOVA being especially sensitive to them. The variables were square-root-transformed prior to the analysis to increase the homogeneity of variance.

The statistical package STATISTICA version 6.0 and SPSS version 14.0 were 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

Chromosome numbers, geographical distribution and ecology

The following chromosome numbers were found in *S. rosmarinifolia*: $2n = 18$, 36, 36+1B, 36+2B. Diploid cytotypes ($2n = 2x = 18$), located in the central and northern Iberian Peninsula in the Occidental and Central Iberian Systems (Fig. 1) are found on a wide

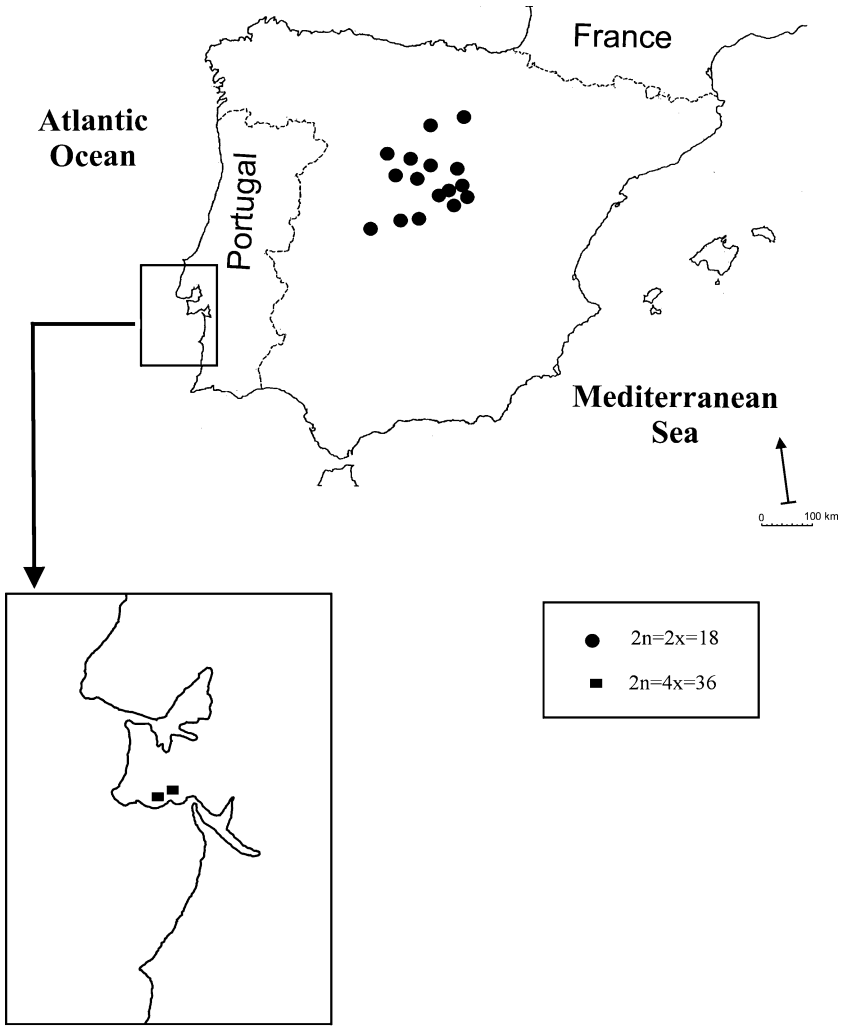


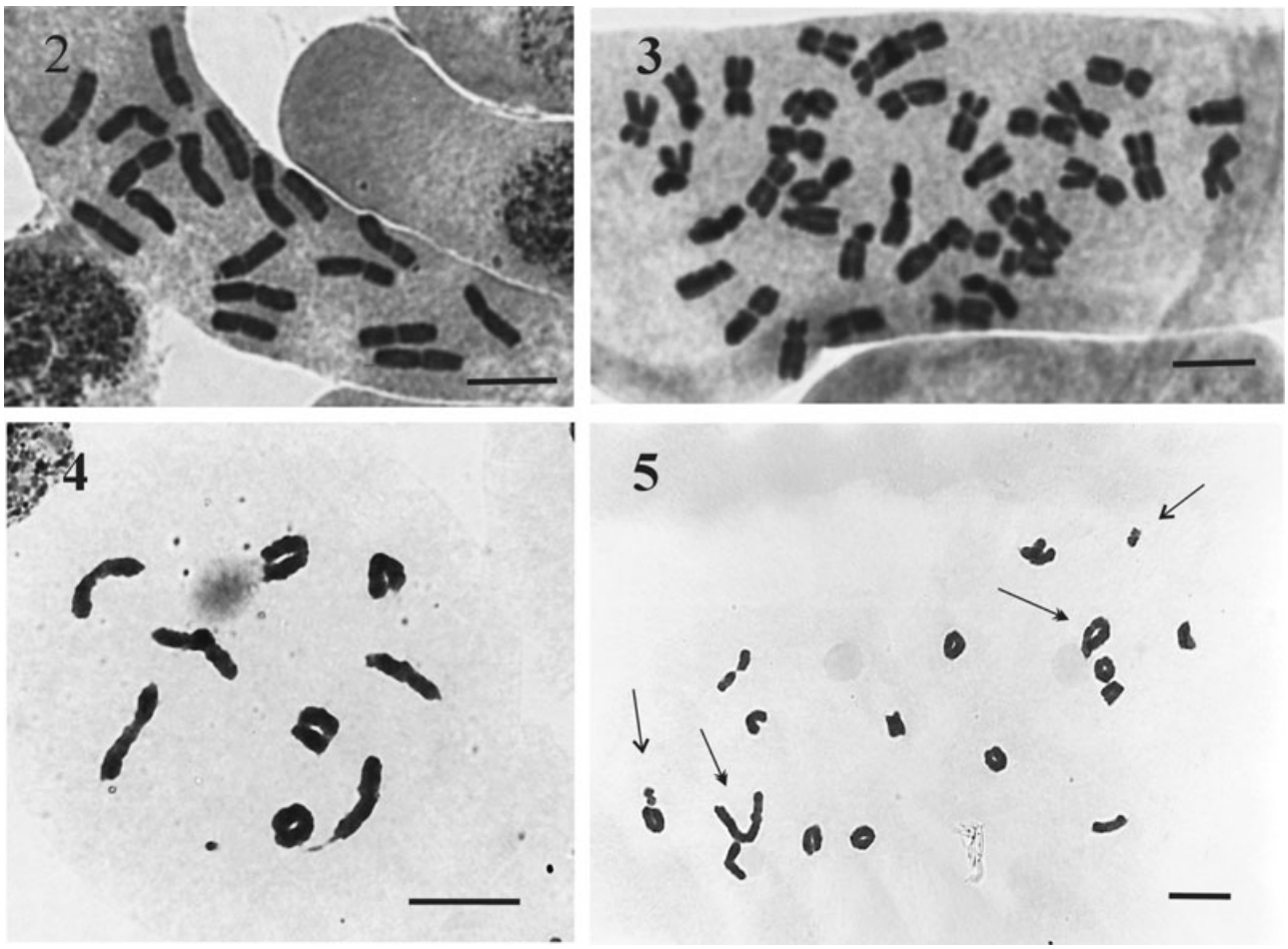
Figure 1. Location of the *Santolina rosmarinifolia* populations studied.

range of substrates at 450 to 2000 m. The two tetraploid populations ($2n = 4x = 36$, 36+1B, 36+2B) were located in Serra da Arrábida, Portugal (Fig. 1), on marl–limestone and sandstone and limestone conglomerate, between 100 and 160 m.

The diploid cytotypes tolerate extremely cold winters (5.2 ± 2.1 °C) and mild summers (18.8 ± 2.3 °C), whereas the tetraploids grow at a higher in winter (11.7 ± 1.8 °C) and summer (22.5 ± 1.5 °C) temperatures than the diploids. Rainfall is variable through the year, the average being 749.4 ± 376.6 and 725.0 ± 231.0 mm in the diploid and tetraploid areas respectively. The monthly average precipitation is greatest during the autumn (305.9 ± 212.8 and 338.2 ± 168.8 mm for diploid and tetraploid areas, respectively) and scarce in summer (88.6 ± 57.0 mm in the diploid vs. 31.0 ± 31.7 mm in the tetraploid). In general, diploid cytotypes occupy a broader ecological spectrum than the tetraploid.

CHROMOSOME MORPHOLOGY

Somatic metaphase chromosomes are shown in Figures 2 and 3. The mean length of the short arm (LBC) (1.99 ± 0.56 μm), intrachromosomal asymmetry index (A1) (0.26 ± 0.04) and the mean of interchromosomal asymmetry index (A2) (0.14 ± 0.04) were significantly higher in the diploid cytotypes than in tetraploids (LBC: 1.68 ± 0.34 μm; A1: 0.35 ± 0.05; A2: 0.11 ± 0.02). Karyotype asymmetry was 2A at both ploidy levels. The nested MANOVA showed statistical heterogeneity between ploidy levels for chromosome morphology (Wilk's $\lambda = 0.21$; $F_{5,30} = 22.73$; $P < 0.0001$; LBC: $F_{1,34} = 10.89$; $P < 0.01$; 60.60% of the total variance; A1: $F_{1,34} = 30.28$; $P < 0.0001$; 66.10% of the total variance; A2: $F_{1,34} = 11.28$; $P < 0.01$; 40.40% of the total variance), except for LBL and LTC $P > 0.05$), whereas the populations with the same ploidy level were not statistically distinguishable from the others (Wilk's $\lambda = 0.34$; $F_{65,145.71} = 0.57$; $P > 0.05$).



Figures 2–5. Somatic metaphase and meiotic configuration in diakinesis of *Santolina rosmarinifolia*. Fig. 2. Somatic metaphase, $2n = 2x = 18$, with chromosome formula 12m+2m^{sat}+2sm^{sat}+2st, population 3. Fig. 3. Somatic metaphase, $2n = 4x = 36$, with chromosome formula 24m+8sm+4st, population 18. Fig. 4. Diakinesis, $2n = 2x = 18 = 3$ OII+6 CII and two bivalents associated with the nucleolus are observed, population 1. Fig. 5. Diakinesis, $2n = 4x = 36+2B = 1$ OIV+1 CIV+7 OII+7 CII+2B, the arrows indicate B metacentric chromosomes, double arrows indicate chain and open ring quadrivalent; two nucleoli are observed, one of them with an open ring quadrivalent and two bivalents associated. Scale bar, 6 μm.

The same analysis with regard to chromosome pairs at each ploidy level showed statistical heterogeneity ($P < 0.0001$) between diploid (Wilk's $\lambda = 0.21$; $F_{36,1040.75} = 19.80$) and tetraploid (Wilk's $\lambda = 0.83$; $F_{4,268} = 12.93$) populations, between individuals nested in diploid (Wilk's $\lambda = 0.09$; $F_{78,1053.41} = 15.82$) and tetraploid (Wilk's $\lambda = 0.08$; $F_{32,989.93} = 28.55$) populations and between chromosome pairs nested in each individual of diploid (Wilk's $\lambda = 0.002$; $F_{936,1056.96} = 7.23$) and tetraploid (Wilk's $\lambda = 0.006$; $F_{320,1073.91} = 8.61$) populations.

The chromosome formula of diploid and tetraploid cytotypes, following the classification of Levan *et al.* (1965), was 12m+2m^{sat}+2sm^{sat}+2st and 24m+8sm+4st, respectively. The post-hoc test showed that the chromosome ratio, which defines each type of chromosome, showed significant differences ($P < 0.0001$) for diploid (for the error term d.f. = 354; mean sum of square = 0.17) and tetraploid cytotypes (for the error term d.f. = 271; mean sum of square = 0.23) between sub-telocentric and metacentric chromosomes only. This indicates that the sub-metacentric chromosomes are not statistically distinguishable from the others.

Meiotic configuration and chiasma frequency

The meiosis in both cytotypes was regular, with a predominance of ring bivalent formation at diakinesis (Table 2). One or two bivalents and one open ring quadrivalent associated with the nucleolus were observed in the diploid (Fig. 4) and tetraploid cytotypes (Fig. 5), respectively. Chiasmata were mostly

Table 2. Frequency and factor loading in PCA of the characters studied in diakinesis of diploid and tetraploid cytotypes of *Santolina rosmarinifolia*

Character	Mean (range) ($N = 151$)	Factor 1 Var (52.26%)	Factor 2 Var (29.18%)	Mean (range) ($N = 54$)	Factor 1 Var (32.85%)	Factor 2 Var (16.82%)	Factor 3 Var (13.58%)
B	–	–	–	1.22 (0–2)	0.24	**0.60**	0.26
I	–	–	–	0.46 (0–4)	0.07	**–0.79**	0.13
CII	4.09 (0–8)	**–0.81**	–0.34	7.92 (0–18)	**0.82**	–0.12	0.32
OII	4.91 (1–9)	**0.84**	0.29	8.29 (0–18)	**–0.91**	0.05	–0.24
OIII	–	–	–	0.07 (0–2)	–0.17	–0.14	–0.07
CIII	–	–	–	0.04 (0–1)	–0.03	**–0.65**	–0.25
CIV	–	–	–	0.27 (0–2)	0.37	**0.50**	–0.32
OIV	–	–	–	0.25 (0–2)	0.27	**0.52**	–0.34
TOT	15.22 (10–23)	0.39	**0.81**	29.31 (15–42)	**–0.94**	0.11	–0.07
TER	11.19 (3–18)	**0.78**	**0.91**	25.87 (11–37)	**–0.89**	0.08	–0.28
PRO	1.26 (0–7)	–0.50	–0.06	1.26 (0–3)	–0.43	0.13	**0.70**
INT	2.76 (0–9)	**–0.73**	0.54	2.19 (0–6)	–0.53	0.15	**0.72**

N, total number of meiocytes studied; Var, cumulative variance (%); B, B chromosomes; I, univalent; CII, rod bivalent; OII, bivalent ring; OIII, trivalent ring; CIV, quadrivalent chain; OIV, quadrivalent ring; TOT, total chiasma frequency; TER, terminal chiasma frequency; PRO, proximal chiasma frequency. Characters with strong-to-moderate correlation with PCA factors are in bold type.

terminal at both ploidy levels. B chromosomes were observed in the tetraploid cytotypes only (Table 2). The nested ANOVA showed statistical heterogeneity between diploid populations (Factor 1: $F_{4,102} = 39.01$; $P < 0.0001$; Factor 2: $F_{4,102} = 2.81$; $P < 0.05$), whereas individuals in the populations were not statistically distinguishable ($P > 0.05$). The reverse occurred in tetraploid cytotypes: the same analysis showed significant differences between tetraploid individuals in the populations (Factor 1: $F_{16,36} = 3.18$, $P < 0.01$; for Factor 2 and Factor 3 $P > 0.05$), whereas the populations were not statistically distinguishable ($P > 0.05$).

Pollen fertility

Pollen was fertile at both ploidy levels. Mean pollen fertility pollen was higher in the diploid cytotypes (94.7 ± 12.9%) than in tetraploids (87.4 ± 12.5%), with a range of 39–100% at both ploidy levels. Pollen fertility showed statistical heterogeneity ($P < 0.0001$) between ploidy levels ($F_{1,138} = 307.28$; 71.00% of total variance) and between individuals in the populations at each ploidy level ($F_{60,138} = 2.78$; 10.70% of the total variance), but not between populations at each ploidy level ($F_{5,138} = 0.52$; $P > 0.05$).

Reproductive fitness

Both cytotypes were fertile, but the mean reproductive fitness was significantly higher in the diploids [NFPC (160 ± 80.21); NAPC (100.52 ± 45.10); PFPC (61.04 ± 30.11)] than in the tetraploids [NFPC (140 ± 62.25); NAPC (80.75 ± 29.52); PFPC (57.10 ± 22.81)]. The mean coefficient of variation of the reproductive fitness characters was higher in diploid (48.10%) than in tetraploid (40.32%) cytotypes.

The nested MANOVA showed statistical heterogeneity ($P < 0.0001$) between ploidy levels (Wilk's $\lambda = 0.39$; $F_{3,303} = 152.61$; NFPC: $F_{1,305} = 60.55$; 46.90% of the total variance; NAPC: $F_{1,305} = 194.11$; 59.90% of the total variance; PFPC: $F_{1,305} = 257.87$; 16.60% of the total variance), between populations at each ploidy level (Wilk's $\lambda = 0.52$; $F_{48,901.99} = 4.50$; NFPC: $F_{16,305} = 69.87$; 25.40% of the total variance; NAPC: $F_{16,305} = 130.96$; 1.40% of the total variance; PFPC: $F_{16,305} = 39.09$; 11.27% of the total variance) and between individuals at each ploidy level and in each population (Wilk's $\lambda = 0.09$; $F_{855,909.96} = 1.32$; NFPC: $F_{285,305} = 6.32$; 17.70% of the total variance; NAPC: $F_{285,305} = 4.17$; 23.70% of the total variance; PFPC: $F_{285,305} = 10.57$; 59.70% of the total variance). Variance components indicated that the variation occurred principally between ploidy levels, except for the fruiting percentage.

Achene germination

Achene weight decreased with achene age, there was a significant increase in time t_0, t_{50} and t_{90} of germination and accumulated germination rate varied significantly ($P < 0.0001$) for all the sources of variation analysed (between ploidy levels: Wilk's $\lambda = 0.12$; $F_{5,92} = 131.05$; between achene age nested in each ploidy level: Wilk's $\lambda = 0.003$; $F_{30,370} = 41.67$; between population nested in ploidy levels and achene age: Wilk's $\lambda = 0.008$; $F_{120,457.04} = 6.18$). Variance components indicated strong significant variation between ploidy levels in relation to achene age (Table 3).

The post-hoc Bonferroni test showed (mean sum of square = 3.13, d.f. = 96) that: (1) the diploid cytotypes showed statistical heterogeneity ($P < 0.0001$) for times t_0, t_{50} and t_{90} and for achene weight between achenes at both 3.2 and 2.2 years with respect to achenes at 0.2 and 1.2 years; (2) tetraploid cytotypes showed significant differences ($P < 0.001$) between achene ages for these parameters, except for achene weight ($P > 0.05$) between achenes at 1.2 and 0.2 years; (3) accumulated germination decreased with achene age, except for the achenes at 1.2 years at both ploidy levels, but no significant variation ($P > 0.05$) was observed between achenes at 0.2 years with respect to achenes at 1.2 and 2.2 years and between achenes at 1.2 years with respect to the achenes at 2.2 years for either cytotype; (4) accumulated germination showed statistical homogeneity ($P > 0.05$) for tetraploid achenes at 1.2 years with respect to diploid achenes at 0.2–2.2 years and for achenes of 3.2 years between the two ploidy levels.

Development and morphology of the seedling

The mean coefficient of variation was higher in diploid cytotypes (38.54%) than in tetraploids (35.77%). The nested ANOVA showed statistical heterogeneity ($P < 0.0001$) between ploidy levels (Factor 1: $F_{1,151} = 1690.83$; 94.30% of the total variance; Factor 2: $F_{1,151} = 0.15$; $P > 0.05$) and between populations at each ploidy level (Factor 1: $F_{5,151} = 9.76$; 1.70% of the total variance; Factor 2: $F_{5,151} = 18.64$; 45.50% of the total variance). These indicate that: (1) tetraploid seedlings were significantly taller, with faster mean leaf growth, larger cotyledons, more lobes per leaf, larger internodes and larger leaves than diploid seedlings; (2) tetraploids showed significantly larger hypocotyls, faster development of the secondary branches and were heavier as a result of having more branches per internode than do diploids (Table 4). Variance components indicated that the variation occurs principally between ploidy levels.

Stepwise discriminant analysis yielded a canonical discriminant function with an eigenvalue of 34.97, high R canonical value (0.98) and $\chi^2_7 = 532.04$, $P < 0.0001$, indicating that the two ploidy levels were significantly different. Factor structure coefficients revealed that plant height (0.53) and length of the internode (0.52) contribute moderately to cytotype differentiation; the coefficient values for the remaining characters were lower than 0.40. The classification matrix indicated that 100% (97 individuals) and 94.73% (54 individuals) of the diploid and tetraploid individuals, respectively, were well classified. This analysis, together with cluster analysis (Fig. 6), indicates that three diploid individuals had patterns of variation for seedling morphology and development similar to those of tetraploids.

MORPHOLOGY OF THE NATURAL POPULATIONS

Quantitative characters

The coefficient of variation of vegetative characters was higher than that of the floral characters. The mean coefficient of variation for vegetative characters was higher in diploid cytotypes (47.94%) than in tetraploids (44.83%). The mean coefficient of variation for reproductive characters was higher in diploid cytotypes (15.27%) than in tetraploids (10.72%). Leaf lobe numbers for flowering and vegetative stems and lobe length of the leaves for flowering stems were the characters with the highest coefficient of variation for both ploidy levels, but the variation was greater in diploid cytotypes (Table 5). Lobe number of the middle leaves for the flowering stems and length of the interseminal bracts were the characters with the highest correlation coefficient with PCA factors (Table 5). These characters had higher values in the tetraploid cytotypes than in the diploids.

PCA factors showed significant differences for all the sources of variation analysed (Table 6). Variance components indicated that the variation occurred mainly between ploidy levels and the variance between individuals in the populations was higher than the variance between populations at each ploidy level.

Stepwise discriminant analysis yielded a canonical discriminant function with an eigenvalue of 8.63, high R canonical value (0.94) and $\chi^2_{78} = 1055.55$, $P < 0.0001$, indicating that the two ploidy levels were significantly different. Factor structure coefficients revealed that the lobe numbers of the lower (–0.23), middle (–0.33) and upper leaves (–0.26) of the flowering stems, the lobe numbers of the lower (–0.23) and middle leaves (–0.13) of the sterile stems, the base length of the interseminal bracts (–0.21) and the central achene width (–0.26) showed a low correlation with the discriminant function, indicating that no character contributed strongly to cytotype differentiation and the lobe number of the middle leaves of the flowering stems was the character with highest contribution to cytotype differentiation.

Table 3. Summary of germination variables and achene weight of diploid and tetraploid cytotypes of *Santolina rosmarinifolia* and their variability by means of nested MANOVA univariate analysis aimed at detecting variation within and between ploidy levels with regard to achene age

Source	Age (years)	AWG Mean ± SD	t_0 Mean ± SD	t_{50} Mean ± SD	t_{90} Mean ± SD	GPA Mean ± SD
2*x*	0.2	1.30 ± 0.50	2.13 ± 0.61	3.77 ± 1.76	9.29 ± 3.55	22.83 ± 1.71 (91.33)
	1.2	1.29 ± 0.52	2.79 ± 0.59	4.33 ± 1.55	9.38 ± 5.50	23.63 ± 1.38 (94.50)
	2.2	1.00 ± 0.60	3.29 ± 0.55	4.96 ± 1.49	7.65 ± 2.97	22.13 ± 2.19 (88.50)
	3.2	0.77 ± 0.32	9.67 ± 2.10	15.13 ± 4.02	27.29 ± 3.52	12.54 ± 2.70 (50.16)
4*x*	0.2	1.37 ± 0.51	2.75 ± 0.89	5.44 ± 1.59	10.13 ± 2.00	18.63 ± 2.56 (74.50)
	1.2	1.35 ± 0.50	5.13 ± 0.35	7.88 ± 1.30	14.19 ± 3.56	21.38 ± 1.41 (85.50)
	2.2	1.00 ± 0.58	6.88 ± 1.25	10.44 ± 1.47	13.88 ± 0.88	19.00 ± 4.14 (76.00)
	3.2	0.65 ± 0.41	17.13 ± 2.47	23.13 ± 2.49	27.63 ± 4.32	12.38 ± 2.39 (49.50)
BNP (d.f.e. = 1; d.f.r. = 96)	F [VCP (%)]	151.59** (0.90)	283.66** (5.90)	127.21** (3.90)	25.70** (3.40)	45.40** (4.00)
ANP (d.f.e. = 6; d.f.r. = 96)	F [VCP (%)]	36.78** (70.80)	296.50** (86.50)	145.42** (83.30)	146.26** (83.00)	122.18** (80.60)
PNA (d.f.e. = 24; d.f.r. = 96)	F [VCP (%)]	35.06** (20.00)	3.44** (2.90)	2.45* (3.40)	4.26** (4.20)	4.10** (4.50)

AWG, achene weight; t_0, t_{50} and t_{90} (number of days until beginning, 50% and 90% of germination was reached, respectively); GPA, accumulated germination rate, average percentage of germinated achenes BNP, between ploidy levels; APP, between achene age nested in ploidy levels; PNA, between populations nested in ploidy levels and achene age; d.f.e., degree of freedom of the effect; d.f.r., degree of freedom of the error term; VCP, variance components (%); SD, standard deviation. *$P < 0.01$; **$P < 0.0001$.

Table 4. Descriptive statistics and factor loading in PCA of diploid and tetraploid seedlings of *Santolina rosmarinifolia*

	Diploid			Tetraploid				
Character	Range	Mean ± SD	CV (%)	Range	Mean ± SD	CV (%)	Factor 1 Var (36.33%)	Factor 2 Var (13.84%)
LCOT*	1.04–3.87	2.61 ± 0.56	21.45	2.80–5.46	4.20 ± 0.61	14.52	**–0.88**	–0.06
ACOT*	0.80–2.69	1.53 ± 0.36	23.52	1.61–2.90	2.12 ± 0.22	10.37	**–0.75**	–0.14
LH*	2.16–50.95	6.18 ± 2.42	39.15	11.05–52.01	28.66 ± 5.39	18.8	**–0.75**	–0.48
NL	0.00–120.00	16.11 ± 13.82	85.78	0.00–205.00	21.71 ± 20.92	96.36	–0.35	0.44
DAH*	0.00–8.01	2.31 ± 1.14	49.35	0.00–7.78	2.17 ± 1.61	74.19	0.03	**–0.87**
LLH*	0.00–7.37	1.26 ± 0.67	53.17	0.00–11.72	1.22 ± 1.06	86.88	0.03	**–0.61**
MCO‡	55.00–60.00	58.00 ± 0.47	0.81	55.00–60.00	58.50 ± 0.58	0.99	0.02	0.12
AGS‡	6.00–10.00	7.16 ± 2.63	36.73	5.00–8.00	6.40 ± 1.97	30.78	**0.50**	–0.25
RAM‡	41.00–48.00	44.71 ± 1.50	3.35	50.00–55.00	51.28 ± 0.45	0.87	**–0.55**	–0.13
ENT*	0.12–3.57	1.42 ± 0.69	48.59	3.75–16.00	7.89 ± 2.37	30.03	**–0.88**	–0.04
LHIP*	3.79–14.46	9.16 ± 2.57	28.05	2.55–21.62	7.67 ± 3.41	44.45	0.28	–0.46
ALP*	10.01–45.00	18.27 ± 6.45	35.3	30.00–75.00	46.27 ± 10.87	23.49	**–0.87**	0.00
WGH†	0.11–0.95	0.29 ± 0.22	75.86	0.10–0.32	0.18 ± 0.06	33.33	**0.55**	–0.18

For character code see Appendix 1. SD, standard deviation; CV, coefficient of variation; Var, cumulative variance. Characters with strong-to-moderate correlation with PCA factors are in bold type. *in mm; †in g; ‡in days.

Cluster analysis (Fig. 7) indicated high similarity among diploid individuals, whereas tetraploid individuals showed high dissimilarity among themselves and with diploid individuals.

Qualitative characters

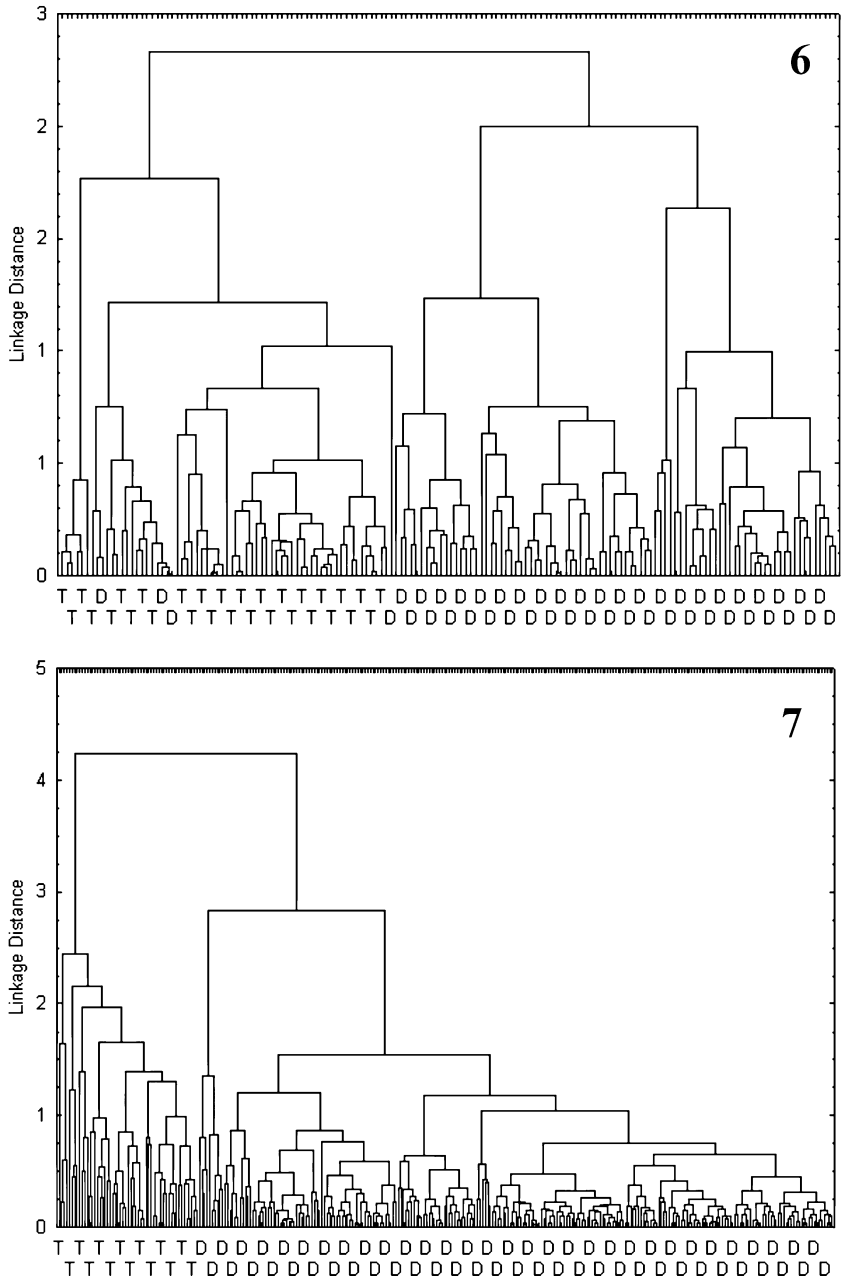
Qualitative morphological characters that differentiate the two cytotypes are shown in Table 7. Multidimensional scaling showed that the characters had a higher value with dimension 1, whereas appendage insertion of the outer bracts showed a moderate value with dimension 2 (Table 7). Cronbach's alpha (D1 = 0.93; D2 = 0.46) and eigenvalue (D1 = 7.19; D2 = 1.10) indicate that the characters correlated with dimension 1 were more important for cytotype discrimination. Dimensions showed significant differences for all the sources of variation analysed (Table 6). Variance components indicated that the variation occurred mainly between ploidy levels and the variance between individuals in the populations was higher than the variance between populations at each ploidy level. Logistic regression analysis showed significant differences between ploidy levels for these qualitative characters (Table 7). The results also indicated that all diploid and tetraploid individuals were well classified. These characters contributed strongly to cytotype differentiation (Fig. 8).

DISCUSSION

Cytogenetics

The results of this study show that the basic chromosome number of *S. rosmarinifolia* is $x = 9$, which agrees with that proposed for the genus by Valdés-Bermejo & Antúnez (1981) in the karyotype study of the Spanish species of the genus. Fernández & Queirós (1971) studied only one individual of the Azeitão population and this was a hypotetraploid. This work demonstrates that both tetraploid populations have the same chromosome number. Probably, the hypotetraploid individual arose by fusion of abnormal gametes originating from the non-disjunction of chromosomes during meiotic anaphase (Fernández & Queirós, 1971; Lacadena, 1996; Singh, 2003) in the tetraploid and perhaps the frequency of hypotetraploids is low in this population.

The karyotype morphology of the diploid and tetraploid cytotypes is similar to that found by Valdés-Bermejo & Antúnez (1981) and Fernández & Queirós (1971), respectively. Chromosome doubling produces a statistically significant decrease in the length of the short arm, the long arm and the whole chromosome, in agreement with the results of Franklin de Melo *et al.* (1997) in Velloziaceae and Solis Neffa & Fernández (2000) in *Turnera* (Turneraceae). However, dif-



Figures 6–7. UPGMA dendrogram constructed with morphological data of diploid (D) and tetraploid (T) cytotypes of *Santolina rosmarinifolia* derived from analysis of the distance matrix of 158 and 305 OTUs of the seedling and of the natural populations, respectively. Fig. 6. Seedlings. Fig. 7. Natural populations.

ferences between the cytotypes with regarding to chromosome characteristics are not great. The karyotypes show low values of asymmetry, as is common in the tribe Anthemideae (Schweizer & Ehrendorfer, 1983).

Tetraploid cytotypes show a tendency to form mostly bivalents. This allows a more complete and rapid segregation and recombination of characters than predominant multivalent formation during meiosis and should give a more normal and balanced distribution of the chromosomes into reproductive cells and, thereby, improve fertility.

B chromosome frequency per meiocyte is low in tetraploid populations, even though the number of meiocytes with B chromosomes observed was high and no significant effects on homologous pairing or on pollen fertility were detected. The B chromosomes could originate from centromeric fragments (Jackson,

Table 5. Descriptive statistics of the quantitative characters of the natural populations of diploid and tetraploid cytotypes of *Santolina rosmarinifolia* that show moderate correlation (in bold type) with first two factors of PCA

	Diploid			Tetraploid				
Character	Range	Mean ± SD	CV (%)	Range	Mean ± SD	CV (%)	Factor 1 Var (11.82%)	Factor 2 Var (7.77%)
LHIF*	3.87–41.36	17.20 ± 5.20	30.23	8.47–56.56	23.07 ± 9.10	39.44	**–0.54**	0.05
LHIV*	6.77–62.79	20.80 ± 7.30	35.09	9.58–60.62	31.27 ± 9.70	31.02	**–0.56**	–0.12
LHB*	4.50–25.60	13.75 ± 3.21	23.34	5.50–34.58	18.10 ± 4.75	26.24	**–0.58**	–0.07
LHC*	4.56–24.54	12.28 ± 2.88	23.45	5.46–35.22	17.44 ± 4.35	24.94	**–0.58**	–0.07
AHSF*	0.35–3.16	1.15 ± 0.35	30.43	0.48–2.02	0.94 ± 0.29	30.85	–0.05	**–0.51**
AHIV*	0.40–3.00	0.92 ± 0.34	36.95	0.60–3.57	1.49 ± 0.60	40.26	**–0.51**	–0.22
AHMV*	0.44–2.70	0.96 ± 0.28	29.16	0.31–3.40	1.38 ± 0.70	50.72	**–0.53**	–0.16
NLHIF	0–38	3.37 ± 5.00	148.36	0–76	20.71 ± 16.04	77.45	**–0.55**	–0.22
NLHIV	0–50	4.48 ± 5.66	126.34	2–71	24.17 ± 13.93	57.63	**–0.50**	–0.30
NLHMF	0–54	2.70 ± 6.23	230.74	0–82	31.69 ± 19.81	62.51	**–0.66**	–0.36
NLHSF	0	–	–	0–40	9.53 ± 11.34	118.99	**–0.53**	–0.40
LLHIF*	0.00–1.30	0.14 ± 0.18	128.57	0.00–1.60	0.43 ± 0.33	76.74	**–0.55**	–0.20
LLHMF*	0.00–1.80	0.06 ± 0.14	233.33	0.00–1.30	0.37 ± 0.28	75.67	**–0.55**	–0.28
LBE*	2.00–4.75	3.17 ± 0.44	13.88	2.00–4.20	2.93 ± 0.43	14.67	–0.23	**0.50**
LBM*	2.00–5.50	3.35 ± 0.46	13.73	2.00–4.50	3.25 ± 0.52	16.00	–0.31	**0.60**
LB1I*	2.00–5.32	3.35 ± 0.46	13.73	2.50–5.60	3.80 ± 0.64	16.75	**–0.62**	0.47
LB2I*	1.86–5.06	3.25 ± 0.46	14.15	2.70–5.40	3.91 ± 0.56	14.32	**–0.63**	0.28
LBP*	2.00–4.00	2.81 ± 0.38	13.52	2.50–4.70	3.60 ± 0.42	11.66	**–0.68**	–0.25
AAQP*	0.40–0.81	0.65 ± 0.07	10.76	0.60–1.00	0.78 ± 0.08	10.25	**–0.54**	–0.31

For character code see Appendix 1. SD, standard deviation; CV, coefficient of variation; Var, cumulative variance; *in mm.

Table 6. Morphological variability within and between ploidy levels of *Santolina rosmarinifolia* by means of nested ANOVA (d.f.r. = 305)

Source of variation	d.f.e.	Quantitative characters		Qualitative characters	
		Factor 1	Factor 2	D1	D2
ENP	1	6955.09* (82.30)	1078.55* (34.00)	30 683.49* (93.70)	141 023.10* (99.00)
EPP	16	42.29* (1.50)	68.61* (8.20)	156.92* (2.10)	29.60* (0.40)
EIN	285	16.29* (14.30)	20.54* (52.40)	16.10* (3.60)	11.70* (0.60)

ENP, between ploidy level; EPP, between populations at each ploidy level; EIN, between individuals in the population and ploidy level; d.f.e., degree of freedom of the effect; d.f.r., degree of freedom of the error term; *$P < 0.0001$. Variance components in parentheses.

1965; Palestis *et al.*, 2004) or by deletion of chromosome arms (Jones, 1991).

The grouping of the chromosomes into fours and the large number of quadrivalents formed at meiosis, together with the close morphological similarity observed between diploid and tetraploid *S. rosmarinifolia*, suggest that the tetraploid is an autopolyploid. Although no unreduced gametes were found in the diploid populations studied, the sexual mechanism of tetraploid formation through bilateral fusion of unreduced gametes (Harlan & de Wet, 1975; Bretagnolle, 2001) is plausible. However, non-reduction is rare, so the chance of a non-reduced pollen grain fertilizing a non-reduced egg cell is low, particularly in view of the large numbers of fertile haploid gametes that are produced by diploids (P. E. Brandham, pers. comm.); more probably it is somatic doubling or a two-step non-reduction process involving a triploid bridge (Husband, 2004). Nevertheless, the high frequency of bivalents suggests that the tetraploid cytotypes of *S. rosmarinifolia* may arise by allopolyploidy. In addition, the study of variation and covariation within and between 12 taxa of *Santolina* genus (A. O. Rivero-Guerra, unpub. data) indicate that polyploidy and hybridization may be a highly effective evolutionary mechanism for producing new plant species, promoting their persistence and survival and ultimately increasing the diversity of plant species. This polyploidy shows 98.18% and 65.45% of the individuals well classified for quantitative and qualitative characteristics, respectively (A. O. Rivero-Guerra, unpubl. data). However, qualitative characters of the seedling are in agreement with the development of diploid cytotypes of *S. rosmarinifolia*.

Pollen fertility and reproductive fitness

In agreement with the results of Bretagnolle & Lumaret (1995) in *Dactylis glomerata* L. subsp. *lusitanica* Stebbins & Zohary (for pollen fertility), Burton & Husband (2000) in *Chamerion angustifolium* (L.) Holub (for reproductive fitness), Ahanchede, Poirier-Hamon & Darmency (2004) in *Setaria italica* P.Beauv. and Rivero-Guerra (2008) in *Santolina pectinata* Benth., pollen fertility and reproductive fitness in *S. rosmarinifolia* were lower in tetraploid cytotypes than in diploids, although these characters had higher values in *S. rosmarinifolia* than in *S. pectinata*. However, Bretagnolle & Lumaret (1995) and Burton & Husband (2000) found that the pollen fertility was similar in diploid and tetraploid cytotypes of *D. glomerata* L. subsp. *lusitanica* and *C. angustifolium*, respectively, whereas Mooring (1994) demonstrated that average pollen stainability was lower in diploid than in tetraploid populations of *Eriophyllum confertiflorum* A.Gray, but the difference was not significant.

Achene germination

In agreement with the suggestion of Levin (2002) and the results of Datta (1963) in *Corchorus olitorius* L. and *C. capsularis* L. and of Hacker (1988) in the *Digitaria milanjiana* Stapf complex, achenes of tetraploid cytotypes of *S. rosmarinifolia* germinate more slowly and have a lower germination percentage than those of diploid cytotypes. The achenes remain viable at 3.2 years at both ploidy levels, but at this age the ability of achenes to germinate decreases significantly, presumably as a result of reduction in achene weight after long-term dry storage at room temperature.

Development and morphology of the seedling

Seedling development is heteroblastic with regard to the morphology of adult plants at both ploidy levels. This work demonstrates that autopolyploids have a significant effect on the morphology and development of *S. rosmarinifolia* seedlings, but the mean value of the coefficient of variation is higher in diploid cytotypes than in tetraploids. Von Well & Fossey (2002)

Table 7. Qualitative morphological characters differentiating diploid and tetraploid cytotypes of *Santolina rosmarinifolia*. Component loading of multiple correspondence analysis and results of logistic regression analysis (d.f. = 1)

Character	Diploid	Tetraploid	D1	D2	χ^2
PLC	Plant usually bright dark green or with yellowish–green flowering stems and dark-green leaves, sterile stems and leaves of the sterile stems usually greyish–glaucous or dark green	Plant, usually with bright olive–green or yellowish–green stems with bright olive–green leaves	0.97	–0.10	348.36**
PUB	Usually glabrous, rarely tomentose to glabrescent	Glabrous or tomentose to glabrescent	0.60	0.15	112.54**
PDS	Thickened above	Usually not thickened or slightly thickened above	0.98	–0.23	251.65**
MHMTF	Usually entire, scaly–dentate or dentate	Generally scaly–dentate, pinnatifid to dentate, dentate or tuberculate–dentate, rarely entire	0.70	0.21	18.38**
LIMTF	Lobes along upper 1/3 to 1/2	Lobes along upper 1/2 to 2/3 on both sides to the margin	0.75	0.25	348.13**
CPS	Usually hemispherical or subglobose	Usually subglobose, or hemispherical	0.98	–0.12	348.36**
CPU	Strongly umbilicate	Usually not umbilicate, rarely umbilicate	0.98	0.13	176.40**
RCP	Usually hemispherical, rarely lenticular	Usually conical, rarely hemispherical	0.98	0.10	259.65**
BEAD	Usually not decurrent or decurrent upper 1/3, rarely decurrent narrowly to the base or upper 1/2	Usually decurrent upper 1/3 or not decurrent, rarely decurrent narrowly to the base or upper 1/2	–0.30	0.62	10.65*

For character code see Appendix 1. D1, dimension 1; D2, dimension 2; $^*P < 0.007$; $^{**}P < 0.0001$.

Figure 8. Two-dimensional scatter plot of the multidimensional scaling performed with the complete data set of qualitative characters of: (1) diploid and (2) tetraploid cytotypes of *Santolina rosmarinifolia*. The percentage of variability accounted for by each dimension is indicated in brackets.

and Baack & Stanton (2005) demonstrated that the growth of *Triticum monococcum* L. subsp. *monococcum* and *Ranunculus adoneus* A.Gray, respectively, is faster in the polyploid than in diploid cytotypes; the results found in *S. rosmarinifolia* are analogous, except for the development of cotyledons and secondary branches, and contradict the results of Min & Ying (1995) in *Citrullus vulgaris* Schrad. ex Eckl. & Zeyh. and with the ideas of Levin (2002).

Furthermore, Hroudova & Zakravsky (1993) in *Butomus umbellatus* L. and Zlesak, Thill & Anderson (2005) in *Rosa chinensis* Jacq. var. *minima* Hort., demonstrated that the seedling characters were smaller in the tetraploid than in the diploid cytotypes; the reverse occurs in *S. rosmarinifolia*, except for the length of the hypocotyls. However, in *S. rosmarinifolia*, diploid seedlings are heavier than those of the tetraploid, due to having more branches per node. These results are not in accord with those obtained by Bretagnolle, Thompson & Lumaret (1995) and Hroudova & Zakravsky (1993), who demonstrated that polyploids are heavier and more robust than diploids. In terms of establishment, this means tetraploid seedlings have an advantage over diploid seedlings with regard to growth rate and final size. Diploids make a great vegetative effort in developing high biomass.

Morphological characters of the natural populations

The results show that the mean of morphological characters is generally increased by polyploidy. In agreement with the results of Schwaegerle & Schaal (1979) in *Sarracenia*, Coyle, Sharik & Feret (1982) in *Betula*, Schnabel & Hamrick (1990) in *Gleditsia*, Jain, Rai & Sing (1981) in *Avena* and Thomas *et al.* (2001) in *Oryza*, these two peripheral, isolated tetraploid populations of *S. rosmarinifolia* show lower significant phenotypic variation (only for quantitative characters) than diploid populations. The reverse occurs for qualitative characters, which show high variation at individual level in the tetraploid cytotypes.

The high coefficients of variation for most characters reflect high variation among individuals in *S. rosmarinifolia*. The results are congruent with the conclusions of Herrera (1990, 2001), Armbruster *et al.* (1999), Niklas (1994) and Klimbo *et al.* (2004) with regard to the variance for vegetative characters, which tends to be higher than that for reproductive characters, the latter being evolutionarily more conservative than the former; but the variance of the reproductive fitness in both cytotypes of *S. rosmarinifolia* is greater still. This is probably because of random selection of capitula, without taking into account the age of the branches, so that at the beginning of the flowering season the flowers can develop achenes, whereas at the end of the season the number of achenes per capitulum may be reduced. In general, the variation coefficient is higher in the diploid cytotypes than in the tetraploids, indicating that the quantitative characters are more variable in the former than in the latter.

Cytotype differentiation

This study demonstrates that the geographically isolated tetraploid populations of *S. rosmarinifolia* show significant phenotypic differentiation from the diploid populations. The results suggest a recent diversification process in this species and that the disjunctive distribution of both cytotypes arose from fragmentation or contraction of the species range, preventing gene flow between them and allowing fixation of the chromosomal, ontogenetic and morphological changes, favouring differentiation and allopatric speciation. It is evident that the two tetraploid populations form a distinct taxonomic entity, which is well delimited from geographical, karyological, ontogenetic and morphological standpoints. The results support recognition of the tetraploid cytotypes at the subspecies level, in agreement with the subspecies concept of Stuessy (1990) and of Hamilton & Reichard (1992). The following new subspecies is proposed:

Santolina rosmarinifolia L. subsp. *arrabidensis,* subsp. nova

A *S. rosmarinifolia* L. sensu stricto praesertim differt chromosomatibus tetraploideis; colore vivide olivaceo, raro caulibus floriferis vivide flavivirentibus et foliis olivaceis; planta glabra vel glabrescenti vel tomentosa; pedunculis non incrassatis, raro leviter incrassatis; foliis mediis squamoso-denticulatis vel pinnatifidis vel dentatis vel tuberculato-denticulatis, in 2/3 partibus superioribus vel in dimidio supero plerumque lobulatis, (0–)3–72(–82) lobulos in utroque latere ferentibus, raro integris; foliis superioribus caulium floriferorum plerumque squamoso-denticulatis vel modo imbricato-tuberculato-denticulatis vel integris; capitulo plerumque semigloboso vel hemisphaerico, plerumque non umbilicato, raro ad basin umbilicato; receptaculo plerumque conico, raro hemisphaerico; phyllariis involucralibus exterioribus appendicem apicalem scariosam plerumque in triente superiore decurrentem vel non decurrentem ferentibus; bracteis interseminalibus 2.5–4.7 mm longis. In montibus Lusitaniae Arrabida dictis prope Setubal tantum habitat.

Typus: Portugal: Setúbal: Serra da Arrábida, in front of tile factory, 160 m, on marl–limestone and limestone conglomerate, 15.vii.1997, Leg.: *A. O. Rivero-Guerra* (*217475*, holotype: SEV).

Description: Plant 30–90 (98) × 55–200 (227) cm, with 1–13 branches per stem, usually with bright olive–green or yellowish–green stems with bright olive–green leaves, glabrous or tomentose to glabrescent. Basal and fascicular leaves (5.5) 8.8–25.3 (34.6) × 0.6–1.3 (1.6) mm, subterete, with obtuse–mucronate apex, with 43–165 (270) lobes, imbricate–tuberculate–dentate on both sides, grooved from the apex to the base on both sides or grooved from the apex to the base above and on the lower 1/2 or 1/3 below. Flowering stems (11.5) 16.2–49.8 (50.5) cm, with 0.7–1.8 (2.0) mm in diameter, fragile. Peduncle 17–130 (985) mm, usually not thickened or slightly thickened and not solid above. Sterile stems (2.8) 3.5–26.0 (43.0) cm. Lower and middle leaves (8.5) 9.5–46.2 (60.6) × (0.2) 0.5–2.0 (3.6) mm, with (0) 3–72 (82) lobes of (0) 0.1–1.3 (1.6) mm, with acute–mucronate or obtuse–mucronate apex; the lower pinnatifid–pectinate, pectinate–pinnatisect, pinnatipartite–pectinate or tuberculate–denticulate grooved on both sides; the middle scaly–dentate, pinnatifid to dentate, dentate or tuberculate–denticulate grooved on both sides, rarely entire. Upper leaves of the flowering stems (3.2) 5.10–21.0 (26.8) × 0.5–1.9 (2.0) mm, entire with thickened involute–appressed margin, scaly–dentate or imbricate–tuberculate–denticulate. Lobes elliptical, with acute–mucronate or obtuse–mucronate apex, usually along 1/2 to 2/3 upper on both sides to the margin and entire with thickened involute–

appressed margin 1/2 or 1/3 lower. Capitulum (4.0) 4.8–7.7 (8.0) × (5.82) 8.2–14.8 (15.5) mm, usually subglobose or hemispherical, generally not umbilicate, rarely umbilicate at the base. Receptacle (1.7) 2.2–4.9 (5.1) × 2.3–4.9 (5.4) mm in diameter, usually conical, rarely hemispherical. Involucral bracts 2.0–5.0 (5.6) × (0.7) 1.0–2.8 mm, in four rows, carinate, usually with red tip, with an apical scarious appendage, generally decurrent narrowly to the base or decurrent along the upper 1/3 or 1/2, except for the outer, with an appendage generally decurrent along the upper 1/3; the outer triangular or ovate, strongly carinate from the apex to the base, generally with acute or acuminate apex, with apical appendage 0.10–1.1 × 0.2–1.8 (2.0) mm; the middle triangular, strongly carinate from the apex to the base, with appendage 0.1–1.3 (1.5) × 0.2–2.7 mm; first row of inner bracts elliptical, ovate or oblong, strongly carinate lower 2/3, with apical appendage 0.1–2.5 (2.8) × (0.7) 1.0–5.0 mm; second row of inner bracts usually elliptical or obovate, slightly carinate or carinate in the middle zone, with appendage 0.1–2.9 (3.3) × (0.2) 0.7–4.3 (4.5) mm. Interseminal bracts 2.5–4.7 × (0.8) 1.0–1.9 mm; first row usually obovate or elliptical with truncate or rounded apex; the remaining oblong, with acute apex. Flowers 82–200 per capitulum. Corolla 3.8–4.9 mm. Anthers 2.3–3.1 mm. Style 3.2–4.9 mm. Corolla tube 2.0–3.0 (4.5) mm. Corolla aperture (1.0) 1.2–1.5 (1.7) mm. Corolla lobes 0.5–1.0 mm. Achene 1.2–2.1 (2.4) × 0.5–1.0 mm. Flowering from the middle of June to the end of July.

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APPENDIX 1

MORPHOLOGICAL CHARACTERS STUDIED IN *SANTOLINA ROSMARINIFOLIA*

Seedling characters: LCOT, cotyledon length; ACOT, cotyledon width; LH, mean length of the first twelve leaves; NLH, mean lobe numbers of the first 12 leaves; DAH, mean distance from the apex to the first lobe of the first 12 leaves; LLH, mean lobe length of the first 12 leaves; MCO, number of days that the cotyledons remained green and turgid; AGS, mean growth of the first 12 leaves; RAM, number of days for initiation of branch development; ENT, internode length of the leaf 1 and 2; LHIP, length of hypocotyl; ALS, seedling height; WGH, dry weight of seedlings. **Natural population characters. Quantitative characters**: DPL, plant diameter; APL, plant height. **Stem characters**: NTFP, number of branches stock; LTFP, length of flowering stems; LPT, length of the stem peduncle; DTF, diameter of the flowering stems; LTV, length of sterile stems. **Flowering stem leaf characters**: LHB, basal leaf length; LHIF, lower leaf length; LHMF, middle leaf length; LHSF, upper leaf length; AHB, basal leaf width; AHLF, lower leaf width; AHMF, middle leaf width; AHSF, upper leaf width; NLHB, number of basal leaf lobes; NLHLF, number of lower leaf lobes; NLHMF, number of middle leaf lobes; NLHSF, number of upper leaf lobes; LLHIF, length of lower leaf lobes; LLHMF, length of middle leaf lobes; LLHSF, length of upper leaf lobes. **Sterile stem leaf characters**: LHIV, lower leaf length; LHMV, middle leaf length; LHSV, upper leaf length; LHCV, facicular leaf length; AHLV, lower leaf width; AHMV, middle leaf width; AHSV, upper leaf width; AHCV, fascicular leaf width; NLHLV, number of lower leaf lobes; NLHMV, number of middle leaf lobes; NLHSV, number of upper leaf lobes; NLHCV, number of facicular leaf lobes; LLHIV, length of lower leaf lobes; LLHMV, length of middle leaf lobes; LLHSV, length of upper leaf lobes; NHC/N, number of fascicular leaves per node. **Capitulum characters**: CD, diameter; CA, height. **Receptacle characters**: ID, diameter; IA, height. **Involucral bract characters**: LBE, base length of the outer bracts; LBM, base length of the middle bracts; LB1I: base length of the first row of the inner bracts; LB2I, base length of the second row of the inner bracts; ABE, base width of the outer bracts; ABM, base width of the middle bracts; AB1I, base width of the first row of the inner bracts; AB2I, base width of the second row of the inner bracts; LABE, appendage length of the outer bracts; LABM, appendage length of the middle bracts; LAB1I, appendage length of the first row of the inner bracts; LAB2I, appendage length of the second row of the inner bracts; AABE, apical width of the outer bracts appendage; AABM, apical width of the middle bracts appendage; AAB1I, apical width of the first row of the inner bracts appendage; AAB2I, apical width of the second row of the inner bracts appendage. **Interseminal bract characters**: LBP, base length; ABP: base width. **Reproductive characters. Floral characters**: LANTP, anther length for the outer flowers; LTECP, theca length for the outer flowers; LESTP, style length for the outer flowers; LPP, corolla lobe length for the outer flowers; LCP, corolla length for the outer flowers; LTFP, corolla tube length for the outer flowers; ALFP, corolla aperture for the outer flowers; ATFP, corolla tube aperture for the outer flowers; LANTC, anther length for the inner flowers; LTECC, theca length for the inner flowers; LESTC, style length for the inner flowers; LPC, corolla lobe length for the inner flowers; LCC, corolla length for the inner flowers; LTFC, corolla tube length for the inner flowers; ALFC, corolla aperture for the inner flowers; ATFC, corolla tube aperture for the inner flowers. **Reproductive characters**: Achene characters: LAQP, outer achene length; AAQP, outer achene width; NCQP, number of outer achene ribs; LAQC, inner achene length; AAQC, inner achene width; NCQC, number of inner achene ribs. **Qualitative characters**: PCL, plant colour: (1) bright dark green; (2) bright yellowish–green stems and dark-green leaves; (3) bright olive–green; (4) bright yellowish–green stems and bright olive–green leaves; PUB, plant pubescence: (1) glabrous; (2) tomentose to glabrescent; PDS, peduncle shape: (1) not thickened

above; (2) slightly thickened above; (3) thickened above; MHMTF, incision of the middle leaves of flowering stems: (1) entire; (2) tuberculate–denticulate; (3) scale–dentate; (4) dentate; (5) pinnatifid to dentate; LIMTF, lobe insertion of the middle leaves of flowering stems: (1) along upper 1/3; (2) along upper 1/2; (3) along upper 1/2 on both sides to the margin; (4) along upper 2/3 on both sides to the margin; CPS, capitulum shape: (1) hemispherical; (2) subglobose; (3) campanulate; CPU, capitulum base: (1) not umbilicate; (2) umbilicate; (3) strongly umbilicate; RCP, receptacle shape: (1) hemispherical; (2) conical; (3) lenticular; BEAD, appendage insertion of the outer bracts: (1) not decurrent; (2) decurrent for the upper 1/3; (3) decurrent for the upper 1/2; (4) decurrent narrowly to the base.