*Botanical Journal of the Linnean Society*, 2008, **156**, 657–667. With 10 figures

# Cytogenetics, geographical distribution, and pollen fertility of diploid and tetraploid cytotypes of *Santolina pectinata* Lag. (Asteraceae: Anthemideae)

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*Received 24 October 2006; accepted for publication 24 October 2007*

A cytogenetic study of 62 populations of *Santolina pectinata* in Spain shows the existence of two ploidy levels. The diploid cytotypes with $2n = 18$ occupy the eastern Betic mountains, and the tetraploid cytotypes with $2n = 36$ are located on the spurs of the Iberian System. The former show a much wider ecological spectrum than the latter. Mixed cytotypes were observed in two diploid populations, with one tetraploid in each, showing different karyotypes. Three trisomic individuals were detected, one in a diploid population and the other two in a tetraploid population. Also, three hypotetraploid individuals were detected in a tetraploid population. Polyploidy is shown to be spontaneous and recurrent, promoting partial sterility in the pollen. Structural chromosomal changes, principally translocations, and local speciation through autopolyploidy are the principal factors in the evolution and diversification of this species.  *Botanical Journal of the Linnean Society*, 2008, **156**, 657–667.

ADDITIONAL KEYWORDS: aneuploidy – Compositae – polyploidization – Spain – speciation.

## INTRODUCTION

Geographical context is important in the understanding of speciation, with the isolation of two or more stocks being a primary contributor to plant speciation (Levin, 1993). Geographical isolation of peripheral populations arises from fragmentation or contraction of the range of the species, preventing gene flow from the central populations, and is an important mechanism for allopatric speciation and for the diversification of certain plant groups (Levin, 1970, 1993; Lesica & Allendorf, 1995; García-Ramos & Kirkpatrick, 1997; Gielly, Debussche & Thompson, 2001). The speciation process of these populations is often rapid as a result of the stochastic process and strong selection pressures (Levin, 1993). Although geographical isolation is not a prerequisite for speciation via polyploidy, ploidal shift is a local phenomenon and is sufficient for species formation (Levin, 1993).

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Established mechanisms of polyploid formation and establishment in nature, as well as the adaptive significance of polyploidy, have been studied by Stebbins (1971), Jackson & Hauber (1982), Fowler & Levin (1984), and Stuessy, Weiss-Schneeweiss & Keil (2004), amongst others. The establishment of autopolyploids in the progenies of diploids has been examined by Felber (1991) and Stuessy *et al*. (2004), although the replacement of diploids by polyploids is not a prerequisite for the establishment of polyploids, as they often occur together (Levin, 2002).

The recurrent origin of polyploidy has been documented by Wolf, Soltis & Soltis (1990) in *Heuchera grossularifolia*, by Van Dijk & Bakx-Schotman (1997) in *Plantago media*, and, above all, by Soltis *et al*. (2004) in *Tragopogon*. In some angiosperm groups, the autopolyploids that originate recurrently are called neopolyploids (Levin, 1993; Stuessy *et al*., 2004). The new polyploid species frequently adapts to a new ecological niche; it is able to present a broad spectrum of tolerance and, with it, a new evolutionary

potential (Levin, 1970, 1993, 2002; Lesica & Allendorf, 1995; García-Ramos & Kirkpatrick, 1997; Stuessy *et al*., 2004).

According to Levin (2002), chromosome rearrangement might (or might not) affect chromosome number, and this chromosome variation can alter fertility, depending on the magnitude and number of rearrangements. By contrast, translocations can alter meiotic behaviour, causing the production of unbalanced gametes, which can give rise to the formation of aneuploid individuals. Occasionally, aneuploid individuals originate spontaneously in the progenies of normal diploids and tetraploids (Singh, 2003).

*Santolina pectinata* Lag. is an infrequently studied Iberian–Maghreb endemic that is found principally in the eastern Betic mountains, extending to a spur of the Iberian System, and in north-east Africa, although López Udías, Fabregat & Mateo (1997) considered that this species extends northwards without occurring beyond the Central System of the Iberian Peninsula. This species of Mediterranean shrub grows on calcareous substrates in Morocco at 1500 m above sea level and is usually associated with *Cedrus atlantica* and *Abies pinsapo*. At lower levels, it is associated with *Quercus ilex* and *Q. canariensis* (Lamrani Alaoui & García Novo, 1999; Mateo, 2003).

Valdés-Bermejo & Antúnez (1981) published the only known chromosome count for *S. pectinata*, in a single tetraploid population located in Sierra de Cazorla, and made no reference to diploid populations. Despite the presence of mixed cytotypes within populations being relatively common in certain groups of plants, including Asteraceae (Husband, 2004; Stuessy *et al*., 2004), there is no information available on the coexistence of different cytotypes in *S. pectinata* populations.

The objective of this work was to analyse the chromosomal morphology variation within and between ploidy levels of *S. pectinata*, particularly the intra- and interpopulation variation of meiotic configuration and chiasma frequency at each ploidy level. In addition, an assessment has been carried out of the mechanism of formation of polyploid populations in nature, and their geographical distribution and adaptive significance in this species.

The study answers the following questions. Are there any patterns of variation of karyotype within and between ploidy levels of *S. pectinata*? What are the cytological causes of the formation and establishment of the polyploid populations?

## MATERIAL AND METHODS

### Cytogenetics and pollen fertility

The study of somatic chromosomes was carried out on root-tip meristems obtained *in vitro* from germinating achenes, the latter originating from natural populations in 1996 (Table 1). The root tips were treated with 8-hydroxyquinoline (0.002 M) (Tjio & Levan, 1950) and fixed in Farmer's fluid (Löve & Löve, 1975). In the study of meiosis and pollen fertility, flower buds were fixed in the field (Table 1) in Carnoy's fluid (Löve & Löve, 1975). The root tips and anthers were stained with alcoholic hydrochloric acid–carmine solution (Snow, 1963), and were squashed on slides in 45% acetic acid. The chromosome number and morphology (length of the short arm, length of the long arm, and total length of the chromosome, excluding the satellite), chromosome ratio (length of the long arm/length of the short arm), number of satellites, chromosome formula (according to the terminology of Levan, Fredga & Sandberg, 1964), chromosome asymmetry indices (according to Romero Zarco, 1986), and karyotype asymmetry (following the classification of Stebbins, 1971) were established from the mitotic plates of 62 individuals (see Table 1). In each individual, three metaphase plates with similar degrees of chromosome contraction were studied.

The meiotic configurations (univalent, bivalent, and multivalent frequencies) were determined following the classification of Jackson & Casey (1982); the frequencies of terminal, interstitial, and proximal chiasmata followed the classification of Sybenga (1975). The study was carried out on five to ten individuals ($9.2 \pm 2.38$) per population. In each individual, three to five meiocytes were analysed, except in the aneuploids, in which ten meiocytes per individual were analysed.

Pollen fertility was estimated by counting 300–400 mature pollen grains per plant, using cotton-blue stain (48 h). The total quantity of sterile pollen was estimated as the sum of the number of aborted pollen grains and the number of pollen grains not stained or lightly stained. The pollen grains that showed cytoplasm uniformly stained dark blue were considered to be viable.

### Statistical methods

Meteorological data (rainfall and temperature) were provided by the National Institute of Meteorology for the areas closest to the various study localities over a period of 20 years. The mean and standard deviation for both variables were determined.

The nested multivariate analysis of variance (MANOVA) technique was employed to analyse the following: (1) the variation of the mitotic variables within and between ploidy levels; (2) the intra- and interpopulation variation of meiotic variables and pollen fertility in both cytotypes; and (3) the within-population variation of the meiotic variables between aneuploid and tetraploid individuals. The *post hoc*

**Table 1.** Location, chromosome number, chromosome formula, and number of individuals studied of *Santolina pectinata* populations

| Pop. | NC (NI) CF | Use | Location |
|---|---|---|---|
| 1 | 18 (4) I | F | Albacete: Alcaraz, 38°38′49″N, 2°30′11″W, 959 m, marl and limestone |
| 2 | 18 (5) I | F, M, S | Albacete: Sierra de Alcaraz, Riopar, 38°29′45″N, 2°24′38″W, 910 m, gypsiferous marl |
| 3 | 18 (10) I, 19 (1) II | F, M | Albacete: Sierra de Alcaraz, between Riopar and Siles, 5 km from Siles, 38°23′59″N, 2°33′26″W, 720 m, gypsiferous marl |
| 4 | 18 (8) | M | Ciudad Real: 10 km from Villahermosa toward Alcaraz, 38°44′35″N, 2°46′18″W, 900 m, marl and limestone |
| 5 | 18 (6) I | F | Granada: between Huéscar and Puebla de Don Fadrique, 1 km from Puebla de Don Fadrique, 37°56′36″N, 2°26′33″W, 1164 m, limestone |
| 6 | 18 (5) | M | Granada: Sierra de la Cabrilla, 37°58′19″N, 2°38′25″W, 1410 m, limestone dolomite |
| 7 | 18 (5) | M | Granada: Sierra de Castril, ascending to Cerro Laguna from Huéscar, 37°52′50″N, 2°45′2″W, 1680 m, limestone |
| 8 | 18 (5) | M | Granada: Sierra de la Sagra, 37°56′46″N, 2°35′33″W, 1300 m, limestone |
| 9 | 18 (6) I | F | Granada: Sierra de la Sagra, Cortijos Nuevos, 37°58′51″N, 2°34′24″W, 1320 m, limestone and sandstone |
| 10 | 18 (6) I | F | Jaén: between Jódar and Huesa, 37°48′28″N, 3°9′2″W, 510 m, limestone |
| 11 | 18 (9) I | F | Jaén: between Huesa and Quesada, 1 km from Quesada, 37°50′26″N, 3°5′29″W, 810 m, limestone and marl |
| 12 | 18 (7) I, 18 + Frag III | F, M | Jaén: between Quesada and Pozo Alcón, 37°47′10″N, 3°1′56″W, 1110 m, limestone and marl |
| 13 | 18 (3) I | F | Jaén: between Quesada and Pozo Alcón, Puerto de Tiscar, 37°46′1″N, 3°1′5″W, 1189 m, limestone and marl |
| 14 | 18 (5) I | F | Jaén: between Quesada and Cazorla, 37°51′37″N, 3°3′35″W, 1090 m, limestone dolomite |
| 15 | 18 (8) | M | Jaén: Santiago de la Espada, 38°6′59″N, 2°33′31″W, 1300 m, limestone and marl–limestone |
| 16 | 18 (10) I | F | Jaén: Sierra de Cazorla, between Quesada and El Chorro, 37°52′13″N, 30°3′37″W, 860 m, limestone dolomite |
| 17 | 18 (8) I | F | Jaén: Sierra de Cazorla, between Quesada and El Chorro, 37°53′9″N, 3°3′8″W, 1000 m, limestone dolomite |
| 18 | 18 (5) | M | Jaén: Sierra de Cazorla, between Quesada and El Chorro, 37°53′36″N, 3°2′5″W, 1120 m, limestone and marl |
| 19 | 18 (5) I | F | Jaén: Sierra de Cazorla, between Cazorla and El Tranco, 37°57′58″N, 2°55′17″W, 1260 m, limestone dolomite |
| 20 | 18 (5) I | F | Jaén: Sierra de Cazorla, between Quesada and El Tranco, 37°59′58″N, 2°54′26″W, 1340 m, limestone dolomite |
| 21 | 18 (6) | M | Jaén: Sierra de Cazorla, between Quesada and El Tranco, 38°3′11″N, 2°52′14″W, 1500 m, limestone dolomite |
| 22 | 18 (5) I | F | Jaén: Sierra de Cazorla, between Quesada and El Tranco, 38°15′31″N, 2°57′28″W, 1400 m, limestone dolomite |
| 23 | 18 (4) I | F | Jaén: Sierra de Cazorla, between Cazorla and El Tranco, 5.6 km from Burunchel, 37°56′55″N, 2°57′34″W, 1240 m, limestone dolomite |
| 24 | 18 (10) | M | Jaén: Sierra de Cazorla, source of Borosa River, 37°57′4″N, 2°50′7″W, 690 m, limestone dolomite |
| 25 | 18 (6) I, 36 (1) V | F, M | Jaén: Sierra de Cazorla, Parador Nacional, 37°54′46″N, 2°57′17″W, 1060 m, limestone dolomite |
| 26 | 18 (8) I | F | Jaén: Sierra de Cazorla, between Cerro Cabañas and Pozo Alcón, 22 km from Cerro Cabañas, 37°43′58″N, 2°58′11″W, 1260 m, limestone and marl |
| 27 | 18 (9) | M | Jaén: Sierra de Las Villas, between Mogón and La Fresnedilla, 38°3′59″N, 2°56′18″W, 1130 m, limestone dolomite |
| 28 | 18 (3) I | F | Jaén: Sierra de Las Villas, Bardazoso, 38°6′10″N, 2°51′21″W, 1340 m, limestone dolomite |

**Table 1.** *Continued*

| Pop. | NC (NI) CF | Use | Location |
|---|---|---|---|
| 29 | 18 (10) | M | Jaén: Sierra de Las Villas, 1.8 km from Aguacebas reservoir bridge, 38°2′28″N, 2°57′14″W, 1130 m, limestone dolomite |
| 30 | 18 (8) I | F | Jaén: Sierra de Las Villas, between Mogón and El Tranco, 38°9′3″N, 2°52′44″W, 1210 m, limestone |
| 31 | 18 (4) I | F | Jaén: Sierra de Mágina, 37°43′52″N, 3°30′54″W, 1400 m, limestone |
| 32 | 18 (4) | M | Jaén: Sierra de Mágina, 37°42′36″N, 3°30′35″W, 1000 m, limestone |
| 33 | 18 (11) I | F | Jaén: Sierra de Segura, Hornos, 38°13′18″N, 2°42′50″W, 867 m, limestone and marl–limestone |
| 34 | 18 (5) I | F | Jaén: Sierra de Segura, La Hoya de Cambrón 38°23′41″N, 2°38′23″W, 1100 m, limestone |
| 35 | 18 (7) I, 36 (1) VI | F, M | Jaén: Sierra de Segura, Orcera, 38°19′32″N, 2°40′8″W, 840 m, gypsiferous marl |
| 36 | 18 (11) I | F, S | Jaén: Sierra de Segura, Pontones, 38°23′41″N, 2°38′23″W, 1350 m, limestone dolomite |
| 37 | 18 (8), 18 + Frag IV | F, M | Jaén: Sierra de Segura, Don Domingo, 38°23′41″N, 2°38′23″W, 1530 m, limestone dolomite |
| 38 | 18 (8) I | F | Jaén: Sierra de Segura, Siles, 38°23′18″N, 2°34′54″W, 800 m, gypsiferous marl |
| 39 | 18 (10) | M | Jaén: Sierra de Segura, Segura de la Sierra, 38°17′55″N, 2°38′34″W, 1100 m, limestone dolomite |
| 40 | 18 (4) I | F | Jaén: Sierra de Segura, Yelmo de Segura, 38°15′41″N, 2°39′55″W, 1500 m, limestone |
| 41 | 18 (5) | M | Jaén: Sierra del Pozo, Cortijo de las Acebadillas, 37°51′12″N, 2°56′23″W, 1890 m, bioclastic limestone and conglomerate |
| 42 | 18 (5) I | F | Jaén: Sierra del Pozo, Nava de San Pedro, 37°54′57″N, 2°56′18″W, 1740 m, bioclastic limestone and conglomerate |
| 43 | 18 (5) | M | Murcia: Moratalla, Sierra del Buitre, 38°9′48″N, 1°54′28″W, 1200 m, limestone |
| 44 | 36 (10) | M | Cuenca: Almodóvar del Pinar, 39°44′1″N, 1°54′46″W, 920 m, limestone |
| 45 | 36 (8) V | F | Cuenca: between Almodóvar del Pinar and Puerto de Tórdigas, 39°47′26″N, 1°56′34″W, 1000 m, limestone |
| 46 | 36 (10) VII | F, M | Cuenca: between Puerto de Tórdigas and Cuenca, 40°8′32″N, 2°20′44″W, 1140 m, limestone |
| 47 | 36 (7), 36 + 3B (3) | M | Cuenca: 2 km from La Almarcha, 39°49′29″N, 2°21′8″W, 890 m, clay |
| 48 | 36 (10) | M | Cuenca: between Cuenca and Ciudad Real, 16 km from Villa Escusa de Haro, 39°38′5″N, 2°34′50″W, 880 m, gypsiferous marl |
| 49 | 36 (9) V | F | Cuenca: 5 km from La Almarcha, 39°42′41″N, 2°22′29″W, 920 m, clay |
| 50 | 36 (10), 36 + 1B* | S | Cuenca: between Almarcha and Cuenca, at Belmontejo crossroads, 39°43′55″N, 2°21′25″W, 850 m, clay |
| 51 | 36 (10) | S | Cuenca: 5 km from Mota del Cuervo towards Cuenca, 39°30′58″N, 2°50′47″W, 740 m, gypsiferous marl |
| 52 | 36 (8), V | F | Cuenca: Cuenca to Ciudad Real road, after the detour towards La Almarcha, 39°41′46″N, 2°22′33″W, 840 m, gypsiferous marl |
| 53 | 36 (6) VI, 37 (2) VIII | M | Cuenca: between Villar de Olalla and San Lorenzo de la Parrilla, 39°52′7″N, 2°20′6″W, 910 m, limestone |
| 54 | 36 (12) V | F | Cuenca: between Cuenca and Almodóvar del Pinar, at the Olmeda del Rey crossroads, 39°50′6″N, 2°0′56″W, 1090 m, gypsiferous marl |
| 55 | 34 (1), 35 (2), 36 (7) | S | Cuenca: Olmeda del Rey, 39°48′53″N, 2°4′22″W, 910 m, marl |
| 56 | 36 (10) V | F | Cuenca: Valeria, 39°48′55″N, 2°8′24″W, 880 m, marl |
| 57 | 36 (10) | M | Cuenca: Valverde de Júcar, 39°43′43″N, 2°13′10″W, 820 m, limestone and marl |
| 58 | 36 (9) V | F | Cuenca: Olivares de Júcar, 39°45′47″N, 2°20′39″W, 850 m, marl |
| 59 | 3 (8) | M | Cuenca: Huete, 40°8′18″N, 2°41′27″W, 840 m, limestone |

**Table 1.** *Continued*

| Pop. | NC (NI) CF | Use | Location |
|---|---|---|---|
| 60 | 36 (5) V | F | Cuenca: Los Pozuelos, Barchín del Hoyo, 39°39′50″N, 2°4′28″W, 1000 m, limestone |
| 61 | 36 (9) | M | Cuenca: Tarancón, 39°59′23″N, 3°0′32″W, 808 m, limestone |
| 62 | 36 (7) V | F | Cuenca: Villarejo de Fuentes, 39°47′19″N, 2°41′36″W, 900 m, limestone |

Pop, population; NC, chromosome number; NI, individual numbers studied; CF, chromosome formula; M, populations in which chromosome morphology is studied; F, populations in which chromosome formula is determined; S, populations in which meiotic and pollen viability are studied; Frag, acentric fragment; I, 13m + $3m^{sat}$ + 2st; II, aneuploid individual that shows trisomy in pair VII (submetacentric), with chromosome formula 12m + $2m^{sat}$ + 3sm + 2st; III, found in 33.33% of the descendants of one individual, with chromosome formula 14m + $1sm^{sat}$ + 1sm + 2st + 1 acentric fragment; IV, found in 20.00% of the descendants of one individual, with chromosome formula 14m + $1sm^{sat}$ + 1sm + 2st + 1 acentric fragment; V, 26m + $2m^{sat}$ + 3sm + $1sm^{sat}$ + 4st; VI, 1M + 18m + $1m^{sat}$ + 7sm + $1sm^{sat}$ + 8st; VII, 24m + $3sm^{sat}$ + 1sm + (2st + $1st^{sat}$ + 1sm) + 3st + $1st^{sat}$, with pair VIII being heteromorphic; VIII, 20m + $1m^{sat}$ + 5sm + $3sm^{sat}$ + $4st^{sat}$ + 4st, showing trisomy in pair VI.

*Found in one of 34 meiocytes studied.

test for the chromosome ratio was carried out using Bonferroni's method. The simple regression technique was applied to evaluate the effect of multivalent frequency and aneuploidy on the pollen fertility of the tetraploid cytotypes.

The techniques were applied after ensuring that data distribution requirements were met for the following: (1) multivariate or univariate normality (in simple regression analysis) by means of the Shapiro–Wilks contrast; (2) the homogeneity of variances by means of the Barlett–Box contrast in the multivariate models and the Levene test in the univariate models (Dytham, 2003; Grafen & Hails, 2003); (3) the presence of rare values or outliers, which were detected graphically, MANOVA being especially sensitive to them; and (4) the linearity of the observations and of the error term (determined graphically) prior to the simple regression analysis. The square root of pollen fertility was calculated prior to the analysis because of the absence of normality in the distribution.

For the statistical analysis, the statistics package STATISTICA, version 6.0, was used. Results were deemed to be significant if the probability of the null hypothesis was less than 0.05.

## RESULTS

### Chromosome numbers, geographical distribution, and cytotype ecology

The following chromosome numbers are found in *S. pectinata*: $2n = 18$, 19, 18 + 1 fragment, 34, 35, 36, 37, 36 + 1B, and 36 + 3B (as shown in Table 1 and Fig. 1).

Diploid cytotypes have the widest distribution range in the species, and are found mainly in protected mountainous zones with limited human impact. They are located primarily in the eastern Betic mountains, particularly in the mountain complex of Cazorla–Segura–El Pozo–Las Villas in Jaén Province and in the Sierra de Alcaraz in Albacete Province. Isolated populations are found in the north-east of Granada Province (Sierra de Castril, Sierra de la Cabrilla, Sierra de la Sagra, and Puebla de Don Fadrique) and in the Sierra de Mágina in Jaén Province, as well as on the plains south-east of Ciudad Real and in the Sierra del Buitre, in the north of Murcia Province (Table 1, Fig. 1). They grow over a broad range of altitudes, from 510 to 1890 m, the average being 975 ± 191.28 m, and on various substrates (Appendix 1), such as soils derived from limestone, limestone dolomites, marl and limestone, limestone and marl–limestone, bioclastic limestone and conglomerate, gypsiferous marl and limestone, and sandstone (Jordán, 2000).

The tetraploid cytotypes extend to spurs of the Iberian System, occupying mainly the southern part of Cuenca Province. Two tetraploid individuals were found in two diploid populations (Parador Nacional of Sierra de Cazorla and Orcera) (Table 1, Fig. 1). These occupy a more restricted and more disturbed area with great human impact towards the north-east of the distribution range, frequently growing on the embankments of highways, in a narrower altitudinal range of 740–1140 m, with an average of 888 ± 120.55 m. They show a less diverse ecological preference and are located on soils derived from limestone, marl, gypsiferous marl, and clay (Table 1).

The tetraploid cytotypes inhabit an area slightly cooler than that occupied by the diploid cytotypes: annual mean temperatures of 13.81 ± 0.82 °C and 14.39 ± 2.37 °C, respectively. They also grow in an area

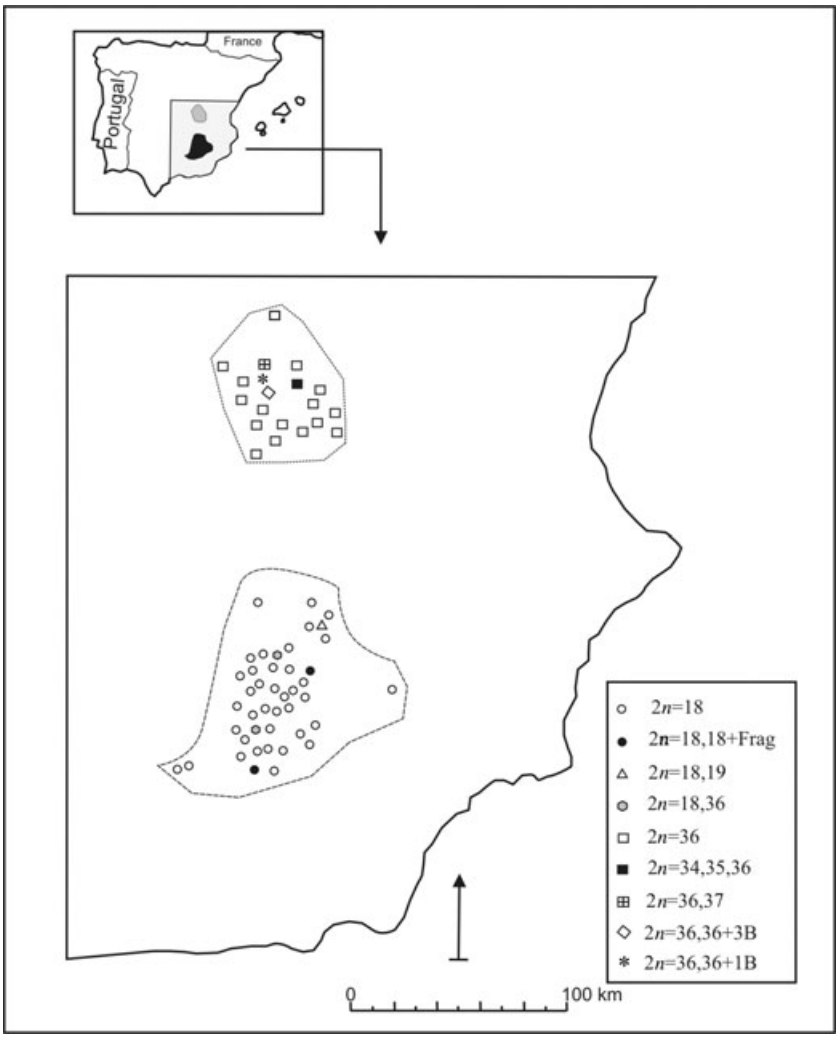



**Figure 1.** Geographical distribution of the diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$) cytotypes of *Santolina pectinata*.

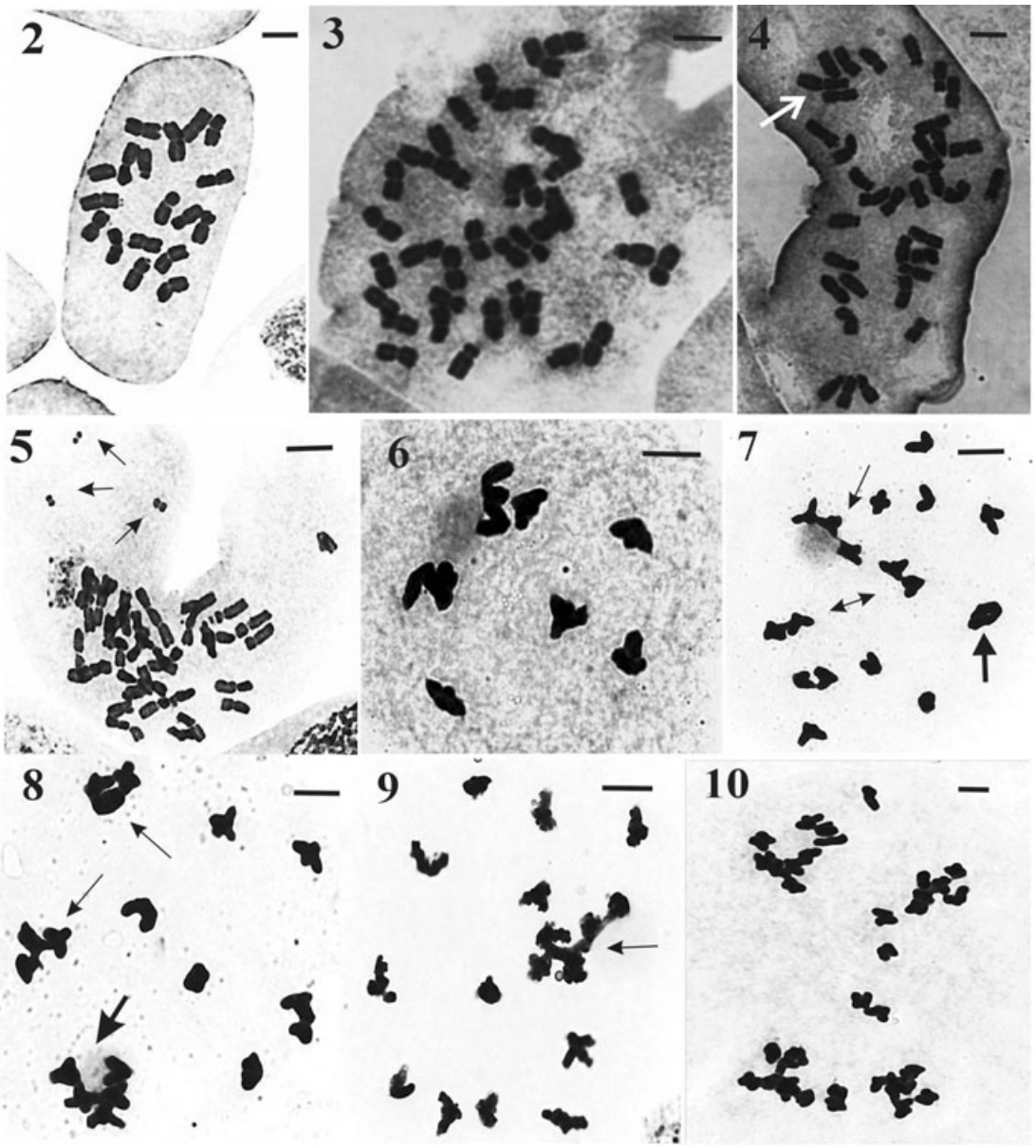
with a lower annual rainfall: averages of 497.38 ± 174.93 mm and 661.73 ± 285.67 mm, respectively.

### Chromosome morphology

The chromosome formulae found in diploid and tetraploid individuals are shown in Table 1. The most common idiogrammatic formulae in diploid and tetraploid cytotypes are $13m + 3m^{sat} + 2st$ and $26m + 2m^{sat} + 3sm + 1sm^{sat} + 4st$, respectively (Figs 2, 3). There is considerable variability in the number of satellite chromosomes in each karyotype, but they are always observed on the shortest arm. In diploid and tetraploid cytotypes, there are 0–4 and 0–8 satellite chromosomes, respectively.

In the mixed population (diploid–tetraploid) of Orcera, the tetraploid individual has an isochromosome (Fig. 4), possibly originating from a Robertsonian translocation between metacentric chromosomes. B chromosomes are observed in two tetraploid populations (Table 1). They are constant in number and much smaller than the A chromosomes (Fig. 5). The average lengths of the long and short arms and the average whole-chromosome length are significantly higher in the diploid than in the tetraploid cytotypes (Table 2). The intrachromosomal asymmetry index ($A_1$) is significantly higher in the tetraploid than in the diploid cytotypes. Chromosomes of the tetraploid populations can be grouped into fours, suggesting that their origin is by autopolyploidy.

Nested MANOVA shows statistical heterogeneity between ploidy levels (Wilk's $\lambda = 0.14$; $F_{4,113} = 171.31$; $P < 0.0001$) and between populations nested in ploidy levels (Wilk's $\lambda = 0.01$; $F_{108,451.12} = 8.36$; $P < 0.0001$). Univariate analysis shows that the intrachromosomal




**Figures 2–10.** Somatic metaphases and meiotic configurations of *Santolina pectinata*. Figure 2. Somatic metaphase, $2n = 2x = 18$ (Albacete: Sierra de Alcaraz, Riopar). Figure 3. Somatic metaphase, $2n = 4x = 36$ (Jaén: Sierra de Cazorla, Parador Nacional). Figure 4. Somatic metaphase, $2n = 4x = 36$. The arrow indicates isochromosome (Jaén: Sierra de Segura, Orcera). Figure 5. Somatic metaphase, $2n = 4x = 36 + 3B$. The arrows indicate B chromosomes (Cuenca: 5 km from La Almarcha). Figure 6. Diakinesis in diploid, 9 CII, four bivalents associated with the nucleolus are observed (Albacete: Sierra de Alcaraz, Riopar). Figure 7. Diakinesis in tetraploid, 1 OII + 9 CII + 1 OIV + 3 CIV. The thin arrow indicates a chain quadrivalent and one bivalent associated with the nucleolus; the thick arrow indicates a ring quadrivalent; the double arrow indicates chain quadrivalents (Cuenca: between Almarcha and Cuenca, at Belmontejo crossroads). Figure 8. Diakinesis, with 9 CII + 3 CVI. The thin arrows indicate two chain hexavalents; the thick arrow indicates a hexavalent chain and two bivalents associated with the nucleolus (Cuenca: Olmeda del Rey). Figure 9. Diakinesis, 1 OII + 11 CII + 1 CXII. The arrow indicates a chain dodecavalent (Cuenca: Olmeda del Rey). Figure 10. Metaphase II in tetraploid, showing several chromosomes between the two groups of dyads, probably originating as laggards at anaphase I (Cuenca: Olmeda del Rey). CII, rod bivalent; CIV, chain quadrivalent; CVI, chain hexavalent; CXII, chain dodecavalent; OII, ring bivalent; OIV, ring quadrivalent. Scale bar: 5 μm (Figs 2–4, 6, 7–10); 7 μm (Fig. 5).

**Table 2.** *Santolina pectinata* karyotype and results of univariate nested MANOVA

| | Diploid | | | Tetraploid | | | Univariate analysis | | |
|---|---|---|---|---|---|---|---|---|---|
| Ploidy level | Range | Mean ± SD (μm) | CV (%) | Range | Mean ± SD (μm) | CV (%) | BPN (d.f. = 1) *F* (VCP) | APP (d.f. = 27) *F* (VCP) | ERR (d.f. = 116) VCP |
| LBC | 1.00–2.81 | 2.81 ± 0.48 | 17.08 | 1.07–2.65 | 2.65 ± 0.43 | 16.22 | 9.31 (6.10)* | 3.59 (32.00)† | 61.90 |
| LBL | 2.89–3.34 | 3.42 ± 0.53 | 15.49 | 2.80–3.66 | 3.28 ± 0.46 | 14.02 | 27.65 (19.60)† | 4.28 (31.80)† | 48.60 |
| LTC | 4.99–6.23 | 5.30 ± 0.97 | 18.30 | 4.46–5.92 | 5.14 ± 0.94 | 18.28 | 29.31 (16.80)† | 5.98 (41.50)† | 41.70 |
| $A_1$ | 0.20–0.31 | 0.27 ± 0.03 | 11.11 | 0.30–0.42 | 0.36 ± 0.12 | 33.33 | 617.03 (58.70)† | 31.57 (35.50)† | 5.80 |
| $A_2$ | 0.10–0.14 | 0.12 ± 0.02 | 16.66 | 0.10–0.16 | 0.12 ± 0.02 | 16.66 | 0.04 (1.23) ns | 15.87 (74.80)† | 23.97 |

$A_1$, intrachromosomal asymmetry index; $A_2$, interchromosomal asymmetry index; APP, amongst populations (ploidy level); BPN, between ploidy levels; CV, coefficient of variation; d.f., degree of freedom; ERR, error; LBC, length of short arm; LBL, length of long arm; LTC, total length of the chromosome; MANOVA, multivariate analysis of variance; SD, standard deviation; VCP, variance components (%); ns, $P > 0.05$.

*$P < 0.01$.

†$P < 0.0001$.

asymmetry index ($A_1$) is a variable with a higher percentage of variance expressed between ploidy levels. Variance components indicate that no particular chromosome character contributes strongly to cytotype differentiation.

The same analysis in relation to chromosome pairs shows statistical heterogeneity ($P < 0.0001$) between ploidy levels (Wilk's $\lambda = 0.62$; $F_{3,394} = 79.42$), between populations nested in ploidy levels (Wilk's $\lambda = 0.07$; $F_{78,1179} = 21.11$), and between chromosome pairs in the populations at each ploidy level (Wilk's $\lambda = 0.004$; $F_{684,1182.93} = 9.17$). Univariate analysis shows significant differences ($P < 0.0001$) for the length of the short arm, length of the long arm, total length of the chromosomes, and chromosome ratio. The *post hoc* test indicates that the chromosome ratio, which defines each type of chromosome, shows significant differences (for the error term d.f. = 396; MS = 0.13; $P < 0.01$) between subtelocentric and metacentric chromosomes only. This indicates that the submetacentric chromosomes are not statistically distinguishable from the others.

## Meiotic configuration, chiasma frequency, and pollen fertility

### *Diploid cytotypes*

Diakinesis of diploid cytotype meiosis is regular (Fig. 6), the chain bivalents (average, 6.80 ± 1.01; range, 4–9) predominating over ring bivalents (average, 2.20 ± 0.95; range, 0–5); these values are based on 60 cells in 20 individuals. The chiasmata are mainly interstitial (Table 3, Fig. 6). The pollen is fertile, the average being 92.69 ± 15.23%, with a range of 33–100%, except for the trisomic individual in population 3, with 67% of its pollen grains being sterile.

Nested MANOVA shows statistical heterogeneity between individuals in populations (Wilk's $\lambda = 0.12$; $F_{55,202.62} = 2.10$; $P < 0.0001$), whereas the populations are not distinguishable statistically (Wilk's $\lambda = 0.79$; $F_{5,43} = 2.21$; $P > 0.05$). Univariate analysis indicates that pollen fertility is the only variable that shows significant differences between individuals in the populations ($F_{11,47} = 9.51$; $P < 0.0001$) and between populations ($F_{1,47} = 9.59$; $P < 0.001$).

### *Tetraploid cytotypes*

Diakinesis of the tetraploid cytotypes is irregular. In addition to univalents, bivalents, quadrivalents, and associations up to dodecavalents are found. The average number of associations per cell, from all individuals studied, is 0.04 (0–1) univalents + 11.52 (5–20) rod bivalents + 2.85 (0–6) ring bivalents + 0.31 (0–2) trivalents + 1.02 (0–4) chain quadrivalents + 0.42 (0–2) ring quadrivalents + 0.81 (0–3) chain

**Table 3.** Chiasma frequency during diakinesis in *Santolina pectinata*

| | Diploid ($N = 58$) | | | Tetraploid ($N = 85$) | | |
|---|---|---|---|---|---|---|
| Variable | Range | Mean ± SD | CV (%) | Range | Mean ± SD | CV (%) |
| QFT | 9–14 | 12.22 ± 1.02 | 8.34 | 14–41 | 28.74 ± 4.66 | 16.21 |
| QFM | 2–9 | 4.95 ± 1.62 | 32.72 | 0–21 | 10.38 ± 4.88 | 47.01 |
| QFI | 4–9 | 7.28 ± 1.31 | 17.99 | 0–34 | 18.33 ± 3.54 | 19.31 |

CV, coefficient of variation; *N*, number of meiocytes studied; QFI, frequency of interstitial chiasmata; QFM, frequency of terminal chiasmata; QFT, total chiasma frequency; SD, standard deviation.

hexavalents + 0.05 (0–1) chain octavalents + 0.04 (0–1) chain decavalents + 0.17 (0–1) chain dodecavalents (based on 85 cells in 27 individuals). Of the associations detected, rod bivalents (Figs 7–10) and chain quadrivalents (Fig. 7), hexavalents (Fig. 8), and dodecavalents (Fig. 9) are those that appear most frequently. Univalents are observed only in a population found between Almarcha and Cuenca, at Belmontejo crossroads. Pentavalent chains and hexavalent rings are observed only in the Olmeda del Rey population. Ten metaphases II are observed, showing several chromosomes between the two groups of dyads, probably originating as laggards at anaphase I (Fig. 10).

Chiasmata are mostly not terminal (Table 3), the majority being proximal, giving rise to the formation of cruciform structures (Figs 7–9). The pollen is partially sterile, the average fertility being 51.95 ± 11.81%, with a range of 20–73%.

Nested MANOVA shows significant differences between populations (Wilk's $\lambda = 0.27$; $F_{26,92.00} = 3.18$; $P < 0.0001$) and between individuals in the populations (Wilk's $\lambda = 0.002$; $F_{312,567.55} = 1.30$; $P < 0.01$). Univariate analysis reveals the following: (1) the frequency of interstitial chiasmata ($F_{2,58} = 7.58$; $P < 0.01$; 10.40% of the total variance) and frequency of hexavalent chains ($F_{2,58} = 4.00$; $P < 0.05$; 4.90% of the total variance) are variables that show statistical heterogeneity at the interpopulation level only; and (2) the frequency of interstitial chiasmata ($F_{24,58} = 2.22$; $P < 0.01$; 60.35% of the total variance), frequency of octavalent chains ($F_{24,58} = 1.77$; $P < 0.05$; 65.32% of the total variance), and percentage of fertile pollen ($F_{24,58} = 2.58$; $P < 0.01$; 66.05% of the total variance) are variables that show statistical heterogeneity between individuals in the populations only.

The results of nested MANOVA of the Olmeda del Rey population show that the meiotic behaviour of the aneuploids does not differ statistically from that of the rest of the tetraploid individuals (Wilk's $\lambda = 0.002$; $F_{66,58.96} = 0.89$; $P > 0.05$). Regression analysis shows that the effects of multivalent frequencies [$N = 84$; $R^2$(adjusted) = –0.007; d.f. = 2; $F = 0.61$; $P > 0.05$] and aneuploidy [$N = 29$; $R^2$(adjusted) = 0.01; d.f. = 1; $F = 0.67$; $P > 0.05$] on pollen fertility are not statistically significant.

## DISCUSSION

The results of this work show that the basic chromosome number of *S. pectinata* is $x = 9$, which agrees with that proposed for the genus by Valdés-Bermejo & Antúnez (1981); however, none of the karyotypes analysed corresponds to that found for a tetraploid individual by Valdés-Bermejo & Antúnez (1981). Trisomic individuals, individuals with acentric chromosome fragments, and hypotetraploid individuals were observed in small and marginal populations, in accord with the suggestion of Levin (2002) that geographically marginal populations can be chromosomally more variable.

Chromosome number doubling produces statistically significant decreases in the lengths of the short arm, long arm, and whole chromosome, in agreement with the results of Franklin de Melo *et al*. (1997) in Velloziaceae and of Solis Neffa & Fernández (2000) in *Turnera* (Turneraceae). However, the differences between the cytotypes with regard to chromosome characteristics are not large. The karyotypes show low values of asymmetry, such as is common in the tribe Anthemideae (Schweizer & Ehrendorfer, 1983).

B chromosomes could originate from centromeric fragments (Jackson, 1965; Palestis *et al*., 2004) or by deletion of chromosome arms (Jones, 1991).

The variation in the number of each type of chromosome is higher in the tetraploid than diploid cytotypes. This is probably produced by translocation or unequal chromosome interchanges. The existence of these processes explains the formation, during meiosis, of configurations above the quadrivalent level in the tetraploid cytotypes. Translocation may also be responsible for the formation of the isochromosome detected in the population of Orcera. Clearly, the karyotype of the tetraploid cytotypes is still in the process of stabilization.

The high sterility of pollen from the trisomic individual detected in the diploid population is indicative of the low tolerance of diploids for aneuploidy, possibly originating from a genetic and physiological imbalance that produces a high index of aborted gametes (Singh, 2003). In the tetraploids, neither multivalent frequency nor aneuploidy affects fertility to any great extent. This has been documented in *Alopecurus* (Sieber & Murray, 1981), although the reverse occurs in *Turnera* (Solis Neffa & Fernández, 2000). As a consequence of delayed and unequal segregation of chromosomes and asynchronous meiosis in the tetraploid cytotypes, abnormal gametes will be produced. They may be responsible for the partial sterility of the tetraploid cytotypes and for the existence of some aneuploid individuals in tetraploid *S. pectinata*.

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. pectinata*, indicate that the tetraploid is an autopolyploid. Although no unreduced gametes were found in the two diploid populations studied, the sexual mechanism of tetraploid formation through bilateral fusion of unreduced gametes (Harlan & deWet, 1975; Bretagnolle, 2001) is plausible. However, nonreduction is very rare, and so the chance that a nonreduced pollen grain will fertilize a nonreduced egg cell falls almost to zero, particularly in view of the large numbers of fertile haploid gametes that are produced by diploids (P. E. Brandham, Royal Botanic Garden, Kew, pers. comm.). More probably, autotetraploidy is derived from somatic doubling or a two-step nonreduction process involving a triploid bridge (Husband, 2004).

The results support the recent origins, spontaneity, and recurrence of the autopolyploid populations of *S. pectinata* from the diploid populations. The uniformly tetraploid populations are located towards the north, and the mixed diploid–tetraploid populations are amongst the diploid populations and isolated from the range of the tetraploids. One is located in the centre of the distribution of diploid cytotypes and the other towards the north-east, close to the limits of their distribution. The tetraploid cytotypes can originate at any point in the diploid geographical area and, at a numerical disadvantage, disperse from the site of origin, potentially establishing as a monotype population without diploid competition, and becoming the focus of dispersion towards disturbed zones with an absence of diploids.

Diploid cytotypes of *S. pectinata* occur in an ecological spectrum broader than that occupied by tetraploid cytotypes. The latter are adapted to disturbed environments, but the former are not. The disjunct distribution of the two cytotypes has anticipated gene flow and allowed the fixation of chromosome changes, which favour karyotype differentiation and consequent allopatric speciation.

## CONCLUSIONS

Variation in the chromosome formula and chromosome number of the *S. pectinata* karyotype indicates that structural changes and local speciation through autopolyploidy are the principal factors of evolution and diversification of this species. The differences between the cytotypes with regard to chromosome characteristics are not large. The polyploidy of *S. pectinata* has been shown to be spontaneous and recurrent, promoting partial pollen sterility. This work demonstrates that diploid cytotypes show an ecological spectrum much broader than that of the tetraploid cytotypes.

## ACKNOWLEDGEMENTS

I wish to thank the Instituto National de Meteorología for information on temperature and rainfall in the study area, and S. Talavera, J. Mejías, M. Laurin, J. Sybenga, and P. E. Brandham for revising and offering suggestions to this work.

## REFERENCES

**Bretagnolle F. 2001.** Pollen reduction and spontaneous polyploidization in diploid populations of *Anthoxanthum alpinum*. *Biological Journal of the Linnean Society* **72:** 241–247.

**Dytham C. 2003.** *Choosing and using statistics. A biologist's guide*, 2nd edn. Oxford: Blackwell.

**Felber F. 1991.** Establishment of the tetraploid cytotype in a diploid population: effect of relative fitness of the cytotypes. *Journal of Evolutionary Biology* **4:** 195–207.

**Fowler NL, Levin DA. 1984.** Ecological constraints on the establishment of a novel polyploid in competition with its diploid progenitor. *American Naturalist* **124:** 703–711.

**Franklin de Melo N, Guerra M, Benko-Iseppon AM, Luiza de Menezes N. 1997.** Cytogenetics and cytotaxonomy of Velloziaceae. *Plant Systematics and Evolution* **204:** 257–273.

**García-Ramos G, Kirkpatrick M. 1997.** Genetic models of adaptation and gene flow in peripheral populations. *Evolution* **51:** 21–28.

**Gielly L, Debussche M, Thompson JD. 2001.** Geographic isolation and evolution of Mediterranean endemic *Cyclamen*: insights from chloroplast *trn*L (UAA) intron sequence variation. *Plant Systematics and Evolution* **230:** 75–88.

**Grafen A, Hails R. 2003.** *Modern statistics for life science*. Oxford: Oxford University Press.

**Harlan JR, deWet JM. 1975.** On Ö. Winge and prayer: the origins of polyploidy. *Botanical Review* **41:** 361–390.

**Husband BC. 2004.** The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. *Biological Journal of the Linnean Society* **82:** 537–546.

**Jackson RC. 1965.** A cytogenetic study of a three-paired race of *Haplopappus gracilis*. *American Journal of Botany* **52:** 946–953.

**Jackson RC, Casey J. 1982.** Cytogenetic analyses of autopolyploids: models and methods for triploids to octoploids. *American Journal of Botany* **69:** 487–501.

**Jackson RC, Hauber DP. 1982.** Autotriploid and autotetraploid cytogenetics analyses: correction coefficients for proposed binomial models. *American Journal of Botany* **69:** 644–646.

**Jones RN. 1991.** B-chromosomes drive. *American Naturalist* **137:** 430–442.

**Jordán A. 2000.** *Mapa litológico de Andalucía*. E. 1 : 100,000. Informe técnico (Exp. 926/98/P/00). Sevilla: Consejería de Medioambiente, Junta de Andalucía.

**Lamrani Alaoui M, García Novo F. 1999.** Etude comparative de la biodiversite des matorrals des Parcs Naturels de Grazalema (Espagne) et de la Talassemtane (Maroc). *Annales de la Recherche Forestiere au Maroc* **32:** 21–43.

**Lesica P, Allendorf FW. 1995.** When are peripheral populations valuable for conservation?. *Conservation Biology* **9:** 753–760.

**Levan A, Fredga K, Sandberg AA. 1964.** Nomenclature for centromeric position on chromosomes. *Hereditas* **52:** 201–220.

**Levin DA. 1970.** Developmental instability and evolution in peripheral isolates. *American Naturalist* **104:** 343–353.

**Levin DA. 1993.** Local speciation in plants: the rule not the exception. *Systematic Botany* **18:** 197–208.

**Levin DA. 2002.** *The role of chromosomal change in plant evolution. Oxford series in ecology and evolution*. Oxford: Oxford University Press.

**López Udías S, Fabregat C, Mateo G. 1997.** *Santolina ageratifolia* Barnades ex Asso (Compositae) y el agregado *S. rosmarinifolia* L. *Anales del Jardín Botánico de Madrid* **55:** 285–296.

**Löve Á, Löve D. 1975.** *Plant chromosomes*. Vaduz: J. Cramer.

**Mateo MA. 2003.** Estudio florístico del Rif Occidental calizo (Marruecos). Unpublished Doctoral Thesis, Universidad de Sevilla.

**Palestis BG, Burt A, Jones RN, Trivers R. 2004.** The distribution of B chromosomes across species. *Cytogenetic and Genome Research* **106:** 151–158.

**Romero Zarco C. 1986.** A new method for estimating karyotype asymmetry. *Taxon* **35:** 526–530.

**Schweizer D, Ehrendorfer F. 1983.** Evolution of C-band patterns in Asteraceae-Anthemideae. *Biologisches Zentralblatt* **102:** 637–655.

**Sieber VK, Murray BG. 1981.** Structural and numerical chromosomal polymorphism in natural populations of *Alopecurus* (Poaceae). *Plant Systematics and Evolution* **139:** 121–136.

**Singh RJ. 2003.** *Plant cytogenetics*, 2nd edn. Boca Raton, FL: CRS Press.

**Snow T. 1963.** Alcoholic hydrochloric acid–carmine as a stain for chromosomes in squash preparation. *Stain Technology* **38:** 9–13.

**Solis Neffa VG, Fernández A. 2000.** Chromosome studies in *Turnera* (Turneraceae). *Genetics and Molecular Biology* **23:** 925–930.

**Soltis DE, Soltis PS, Pires JC, Kovarik A, Tate JA, Mavrodiev E. 2004.** Recent and recurrent polyploidy in *Tragopogon* (Asteraceae): cytogenetic, genomic and genetic comparisons. *Biological Journal of the Linnean Society* **82:** 485–501.

**Stebbins GL. 1971.** *Chromosomal evolution in higher plants*. London: Edward Arnold.

**Stuessy TF, Weiss-Schneeweiss H, Keil DJ. 2004.** Diploid and polyploid cytotype distribution in *Melampodium cinereum* and *M. leucanthum* (Asteraceae, Heliantheae). *American Journal of Botany* **91:** 889–898.

**Sybenga J. 1975.** *Meiotic configuration. Monographs on theoretical and applied genetics*, Vol. 1. Berlin: Springer-Verlag.

**Tjio JH, Levan A. 1950.** The use of oxyquinoline in chromosome analysis. *Anales Estación Experimental Aula Dei* **2:** 21–64.

**Valdés-Bermejo E, Antúnez C. 1981.** Estudios cariológicos en especies españolas del género *Santolina* L. (Compositae). *Anales del Jardín Botánico de Madrid* **38:** 127–144.

**Van Dijk P, Bakx-Schotman T. 1997.** Chloroplast DNA phylogeography and cytotype geography in autotetraploid *Plantago media*. *Molecular Ecology* **6:** 345–352.

**Wolf PG, Soltis DE, Soltis PS. 1990.** Chloroplast-DNA and allozymic variation in diploid and autotetraploid *Heuchera grossulariifolia* (Saxifragaceae). *American Journal of Botany* **77:** 232–244.