Cytogenetics, Geographical Distribution, Pollen Stainability and Fecundity of *Santolina impressa* (Asteraceae: Anthemideae)

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Abstract *Santolina impressa* ($2n = 2x = 18$) is an endemic species of Portugal, with restricted geographical distribution. The present study aimed to explore its chromosomal variation in respect to chromosome morphology, meiotic behaviour, and effects on pollen stainability and fecundity. Its karyotype formula was found to be either $12m + 2m^{sat} + 2sm^{sat} + 2st$ (75% of the individuals) or $12m + 2m^{sat} + 3sm + 1sm^{sat}$ (25% of the individuals). Univalents were observed in 29.21% of the meiocytes. Chromosome fragments due to breakage in the chromosome arm were observed in 10 meiocytes only (11.23% of the meiocytes). Chains and rings of trivalents were observed in 14.60% of the meiocytes (one trivalent per meiocyte was observed). Chains and rings of quadrivalents were observed in 21.34% and 11.23% of the meiocytes, respectively, with a range of 0–1 per cell. Nine plants (40.90% of the total) with $2n = 2x = 18 + 2B$ showed a quadrivalent configuration in diakinesis. Twenty abnormal anaphases with delayed disjunction of the four non-homologous and the two homologous chromosomes were observed. Simple chromosome bridges without fragments and interchromosomal adhesions were observed in 35.95% of the anaphases analyzed. B chromosomes showed bivalent association in diakinesis and their segregation at anaphase I was normal. Pollen was found to be fertile (mean ± s.d. = 89.57 ± 47.14%); the effect of univalent frequency and frequency of abnormal anaphase I on pollen stainability was strong and statistically significant.

Keywords Cytogenetics · Endemism · Fecundity · Nested MANOVA · Pollen stainability

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Introduction

The concept, origin, appearance and expression of the chromosome inversions and translocations in plants were explained in detail by Sybenga (1975). Reciprocal translocations and inversions occur in nature at a low rate, probably in all higher organisms (Grant 1971). Translocation heterozygosity occurs independently of hybridization in some species, where it typically results in reduced fertility (Levin 2002). The presence of chromosome inversions in diploid species has been studied in various taxa. These include *Atriplex longipes* (Gustafsson 1972), *Lolium perenne* (Simonsen 1973), diploid hybrids of *Echeveria* (Uhl 1992), *Helianthus anomalus* (Rieseberg et al. 1995), and *Santolina semidentata*, *S. semidentata* subsp. *melidensis*, *S. canescens,* the hybrid complex of *S. rosmarinifolia* subsp. *rosmarinifolia*, *S. oblongifolia* and their putative hybrids (Rivero-Guerra 2009). In the studied *Santolina* taxa the chromosome inversions have a deleterious effect on pollen stainability. This has been documented also by other authors, e.g., Gustafsson (1972) in *Atriplex longipes*, Sisodia (1971) in *Urochloa*, Spies et al. (1992) in *Tribolium*, Lu (1993) in *Elymus*, Atlagić (1996) in *Helianthus*, and Costa and Forni-Martins (2004) in *Echinodorus tennellus*.

The deleterious effect of univalents on pollen stainability has been documented by Simonsen (1972) in *Phleum pratense*, and by Fiona et al. (1988) in *Solanum muricatum*, while other authors showed that the frequency of univalents is not correlated with pollen stainability. Such studies include those by Dnyansagar and Sudhakaran (1970) in *Vinca rosea*, Merker (1971) in Triticale, Gatt et al. (2000) in some hybrids of *Dahlia*, and Palma-Silva et al. (2004) in *Vriesea* and *Aechmea*.

The *Santolina rosmarinifolia* aggregate comprises nine taxa. Three of them (*S. rosmarinifolia* subsp. *rosmarinifolia* and *S. canescens* (Rivero-Guerra 2009), and *S. pectinata* (Rivero-Guerra 2008a)) have two cytotypes: diploid and tetraploid, while the others are diploid (*S. impressa*, *S. oblongifolia*, *S. semidentata* subsp. *semidentata*, and *S. semidentata* subsp. *melidensis*), tetraploid (*S. rosmarinifolia* subsp. *arrabidensis*; Rivero-Guerra 2008b) or hexaploid (*S. ageratifolia*; Rivero-Guerra 2008c). The taxa of this aggregate show a high degree of sympatry, but *S. rosmarinifolia* subsp. *arrabidensis* and *S. impressa* are isolated in the western part of the *Santolina* range, whereas *S. ageratifolia* occurs in the eastern part of the Iberian Peninsula.

Santolina impressa ($2n = 2x = 18$; Fernández and Queirós 1971) is an endemic species of Portugal. No detailed analyses have been performed previously regarding its chromosome morphological variation, meiotic behaviour, pollen stainability and fecundity. The present paper is a continuation of a series of studies intended to improve our knowledge of the chromosomal variation of this aggregate, and to clarify the taxonomic relationships of these species and their ecology.

The objectives of this work are to explore the following aspects of the biology of *Santolina impressa*: *i*) intra- and interpopulation variation of chromosome morphology, meiotic configuration and the frequency of chiasmata in diakinesis, as well as pollen stainability and fecundity; and *ii*) the effect of univalent frequency and frequency of abnormal anaphase I on pollen stainability.

Material and Methods

Sampling

Six populations of *Santolina impressa* (87 individuals) were collected in the summer of 1998 and analyzed here. Details on localities are given in Table 1, and voucher specimens are deposited in SEV herbarium. The whole distribution area of the *S. impressa* was specified on the basis of available herbarium specimens and the specimens collected by the author. The following herbaria were studied (herbaria abbreviations are according to Holmgren et al. 1990): AH, AK, B, BC, BC-Sennen, BIO, BM, C, CGE, COFC, COI, E, FCO, FI, FR, G, GB, GDA, GDAC, GR, GZU, HAC, HAL, HBG, HEID, ISAL, JACA, JE, K, L, LD, LEB, LINN, LISE, LISU, LIV, LZ, MA, MANCH, MAF, MGC, MO, NY, OXF, P, PAD, PAMP, PH, PO, PR, RAB, REG, SALA, SALAF, SANT, SBT, SEV, SI, TARTU, U, UCLA, UNEX, UPS, UT, VAB, W, WRSL.

Meteorology Data

The meteorological data (rainfall and temperature) were recorded for the areas closest to the studied localities over a period of 20 years, provided by the National Institute of Meteorology of Portugal. The annual mean values and standard deviation for both variables were determined.

Cytogenetics and Pollen Stainability

The study of somatic chromosomes was carried out on root-tip meristems obtained from germinating achenes, collected from natural populations. The root tips were treated with 8-hydroxyquinoline (0.002 M) (Tjio and Levan 1950) and fixed in Farmer's fluid (Löve and Löve 1975). In the study of meiosis and pollen stainability, flower buds were fixed in the field in Carnoy's fluid (Löve and Löve 1975). The root

Table 1 List of the studied populations of *Santolina impressa*. Number of individuals analyzed per population is shown (*N*)

Pop. Nr.	Location	*N*			
		MT	MI	PO	FE
1	Portugal, Comporta, 38°22′46″ N 8°46′28″ W, 14 m	–	–	–	12
2	Portugal, Montevil, 38°23′55″ N 8°36′54″ W, 13 m	5	–	–	13
3	Portugal, Batalha, 38°23′42″ N 8°34′35″ W, 19 m	–	9	9	11
4	Portugal, Troia Peninsula, 38°26′08″ N 8°49′51″ W, 8 m	5	–	–	17
5	Portugal, Torre, 38°20′35″ N 8°46′27″ W 24 m, gravel	5	–	–	7
6	Portugal, Sines, 38°00′05″ N 8°49′46″ W, 34 m, gravel	5	13	13	27

MT – mitosis; MI – meiosis; PO – pollen stainability; FE – fecundity

tips and the anthers were stained with alcoholic hydrochloric acid-carmine solution (Snow 1963) and were squashed individually on slides in 45% acetic acid.

Chromosome number and karyotype morphology (length of the long and short arm, total length of the chromosome excluding the satellite), the chromosome ratio (length of the long arm/length of the short arm), the karyotype formula (according to the terminology of Levan et al. 1965), the chromosomal asymmetry indices (according to Romero Zarco 1986), and the karyotype asymmetry (following the classification of Stebbins 1971) were established from the mitotic plates of 20 individuals of *S. impressa* (Table 1). In each individual, five metaphase plates with similar degrees of chromosome contraction were studied, and their mean was calculated.

The meiotic configurations (univalent, bivalent and multivalent frequencies) were determined following the classification of Jackson and Casey (1982), and frequencies of terminal and interstitial chiasmata followed the classification of Sybenga (1975). The study was carried out in 22 individuals of *S. impressa* (Table 1). In each individual, three to five meiocytes (89 meiocytes in total) were analyzed.

Pollen fertility, indicated by pollen stainability, was estimated by counting 300–400 mature pollen grains in each 22 plants (Table 1), using cotton-blue stain. The total quantity of sterile pollen was estimated as the sum of the numbers of aborted pollen grains and of non- or lightly-stained pollen grains. The pollen grains that showed cytoplasm uniformly stained dark blue were considered fertile.

Fecundity

The numbers of flowers and of achenes per capitulum were determined. For each character, three observations were carried out in 87 individuals (Table 1). The percentage of fruiting was calculated as the number of achenes per capitulum × 100/ number of flowers per capitulum.

Statistical Methods

Each specimen measured was treated as an independent operational taxonomic unit (OTU) for all statistical tests, although dissimilarity between groups of OTUs (populations) was also measured.

A principal component analysis (PCA) was employed to explore the correlation structure of characters studied in meiosis of the natural populations, in order to assess the relative importance of the characters in population differentiation. Nested ANOVA was employed to analyze the variation within and between populations based on the factors of the principal component analysis of meiotic characteristics and of pollen stainability. Nested MANOVA was used to analyze the variation within and between populations of the karyotype characteristics and fecundity.

The *post hoc* test for the chromosome ratios was carried out using Bonferroni's method. The multiple regression technique was applied to evaluate the effects of univalent frequency at diakinesis and of abnormal anaphase I (simple chromosome bridges and delayed disjunction of the four non-homologous and the two homologous chromosomes) on pollen stainability.

The techniques were applied after ensuring that requirements on data distribution were met: *i*) multivariate or univariate normality by means of the Shapiro-Wilk contrast; *ii*) homogeneity of variance by means of the Barlett-Box contrast in the multivariate models and the Levene test in the univariate models (Dytham 2003; Grafen and Hails 2002); *iii*) the presence of rare values or outliers were detected graphically, to which the MANOVA is especially sensitive; *iv*) linearity of the observations and the error term (determined graphically) prior to multiple regression analysis. The characters (except for karyotype characteristics) were square-root-transformed prior to the analysis to increase the homogeneity of variance.

For statistical analysis, the statistical package STATISTICA, version 6.0 (StatSoft, Tulsa, Oklahoma 2005) was used. The correlation coefficient was considered high when $r \geq 0.75$, moderate when $0.50 \leq r < 0.75$, and low when $r < 0.50$. Results were deemed significant if the probability of the null hypothesis was less than 0.05.

Results

Geographical Distribution and Ecology

Santolina impressa is an endemic species of Portugal, with a restricted geographical distribution. Its habitat is formed by dunes (composites of gravel, sand, clay, and mud) from the mouth of the Sado River to Cape Sines (Fig. 1, Appendix 1). It is found at an altitude of 8–34 m, the mean being 18.67 ± 8.21 m. They also grow in an area with a moderate temperature regime, the annual mean being $15.90 \pm 2.00°C$, and low annual rainfall, the annual mean being 444.87 ± 173.89 mm.

Chromosome Number and Morphology

Santolina impressa is diploid ($2n = 2x = 18$). Two karyotype formulae, in accord with the classification of Levan et al. (1965), were found: $12m + 2m^{sat} + 2sm^{sat} + 2st$ (75% of the individuals; Fig. 2a) and $12m + 2m^{sat} + 3sm + 1sm^{sat}$ (25% of the individuals; Fig. 2b). The mean length of the short (2.26 ± 0.53 μm, coefficient of variation CV= 23.45%) and long (3.32 ± 0.90 μm, CV=27.10%) arms of the chromosome, the total length of the chromosomes (5.66 ± 1.40 μm, CV=24.73%), the mean of intrachromosomal asymmetry index ($A_1 = 0.27 \pm 0.03$, CV=11.11%), and the mean of interchromosomical asymmetry index ($A_2 = 0.10 \pm 0.03$, CV=30.00%) showed statistical homogeneity at the interpopulation level (Wilk's $\lambda = 0.19$, $F_{15,33.52} = 1.84$, $P > 0.05$). Karyotype asymmetry was 2A. However, a nested MANOVA in relation to chromosome pairs showed statistical heterogeneity for all the sources of variation analyzed, except for the chromosome ratio at the interpopulation level (Table 2). Variance components indicated that the variation in the length of the long arm and the total length of the chromosome occurs principally between individuals in the populations, whereas the variation in the length of the short arm and chromosome ratio occurs principally between chromosome pairs nested in each population and individual.

The *post hoc* test revealed that the chromosome ratio, which defined each type of chromosome, showed significant differences (for the error term d.f.=180; MS=0.17;
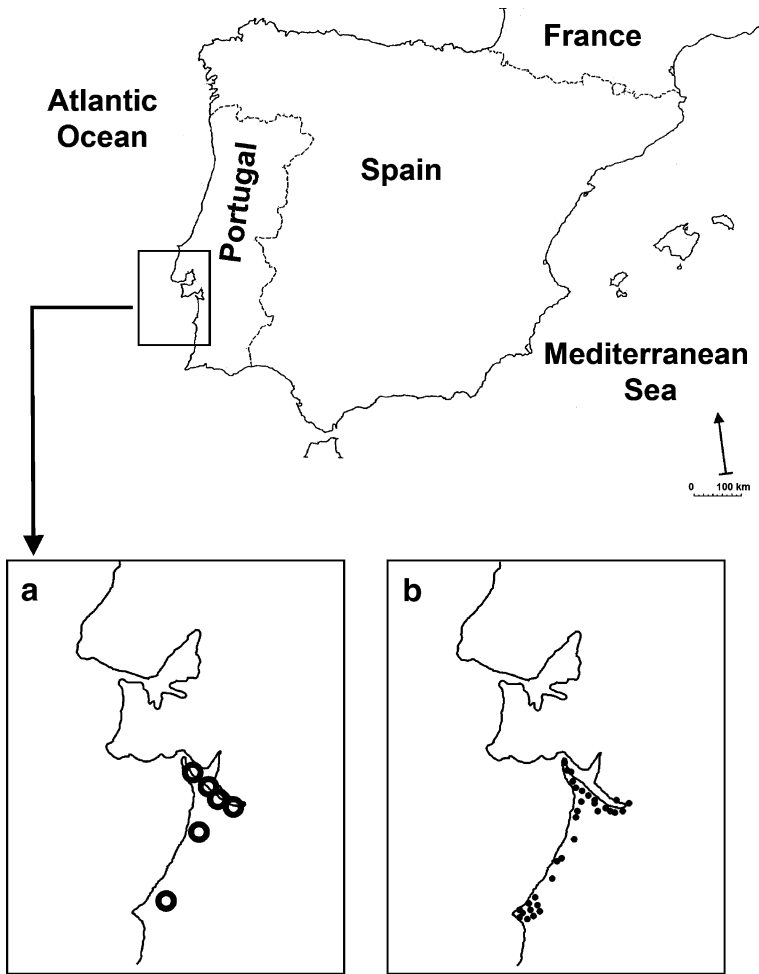
Fig. 1 Geographical distribution of *Santolina impressa* based on the studied populations (**a**) and available herbarium specimens (**b**)

$P<0.0001$) 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 was usually regular; open bivalents (mean ± s.d. = 4.70 ± 2.00) predominated over ring bivalents (mean ± s.d. = 3.06 ± 1.50). Chiasmata were mostly terminal (mean ± s.d. = 10.66 ± 3.57). Interstitial chiasmata are scarcely represented (mean ± s.d. = 0.76 ± 0.56), most being proximal to the centromere, giving rise to formation of cruciform structures (Fig. 2c).

Several meiotic irregularities were observed in *S. impressa* (Figs. 2c–h). Univalents were observed in 45.90% and 10.71% of the meiocytes of Cape Sines

and Bathala populations, respectively, with an average of 2.24±1.59 univalents per cell (Fig. 2d). Chromosome fragments due to breakage in the chromosome arm were observed in 10 meiocytes only (11.23% of the meiocytes) (Fig. 2d). Chains and rings of trivalents were observed in 14.60% of the meiocytes (one trivalent per meiocyte was observed) (Figs. 2d,e). Chains and rings of quadrivalents were observed in 21.34% and 11.23% of the meiocytes, respectively, with a range of 0–1 per cell (Fig. 2f). Nine plants (40.90% of the total) with $2n = 2x = 18 + 2B$ showed quadrivalent configuration in diakinesis (Fig. 2f). Twenty abnormal anaphases I with delayed disjunction of the four non-homologous and the two homologous chromosomes were observed (Fig. 2g). Simple chromosome bridges without fragments and interchromosomal adhesions were observed in 35.95% of the anaphases analyzed (Fig. 2h).

B chromosomes showed bivalent association in diakinesis and their segregation at anaphase I was normal (Fig. 2h). There were no differences in staining potential between A and B chromosomes.

In the PCA analysis the first two factors accounted for 78.06% of the variance. Univalents ($r=0.69$), ring bivalents ($r=-0.78$), open bivalents ($r=0.66$), total chiasma frequency ($r=-0.85$), frequency of terminal chiasmata ($r=-0.73$), chromosome bridges without fragments in anaphase I ($r=0.71$), and delayed disjunction of homologous and non-homologous chromosomes in anaphase I ($r=0.69$) showed moderate to strong correlation with the first factor (Fig. 3). The frequency of interstitial chiasmata ($r=-0.64$) showed moderate correlation with the second factor (Fig. 3). Nested ANOVA showed statistical homogeneity ($P>0.05$) between populations (factor 2: $F_{1,46}=3.68$), and between individuals in each population (factor 1: $F_{20,46}=0.99$; factor 2: $F_{20,46}=0.70$). The same analysis showed statistical heterogeneity ($P<0.0001$) between populations for factor 1 ($F_{1,46}=22.92$; 40.10% of the total variance).

Pollen Stainability

The pollen was found to be fertile, the mean being 89.57±47.14% (CV=52.62%), with a broad range of variation (47–98%). Nested ANOVA showed statistical heterogeneity between populations ($F_{1,46}=4.26$, $P<0.05$; 10.70% of the total variance) and between individuals in each population ($F_{20,46}=17.58$, $P<0.0001$; 84.30% of the total variance). Variance components indicated that the variation within populations was greater.

Multiple regression analysis showed that the effect of univalent frequency and frequency of abnormal anaphase I (simple chromosome bridges and delayed disjunction of the four non-homologous and the two homologous chromosomes) on pollen stainability was strong and statistically significant (adjusted $R^2=0.79$; $F_{3,64}=84.70$; $P<0.0001$). This indicates that the pollen stainability of individuals with meiotic irregularities is lower than that of individuals with normal meiosis, as might be expected.

Fecundity

Flower number per capitulum showed a wide range of variation (90–300 flowers), the mean being 217.89±72.45. Achene number per capitulum and percentage of
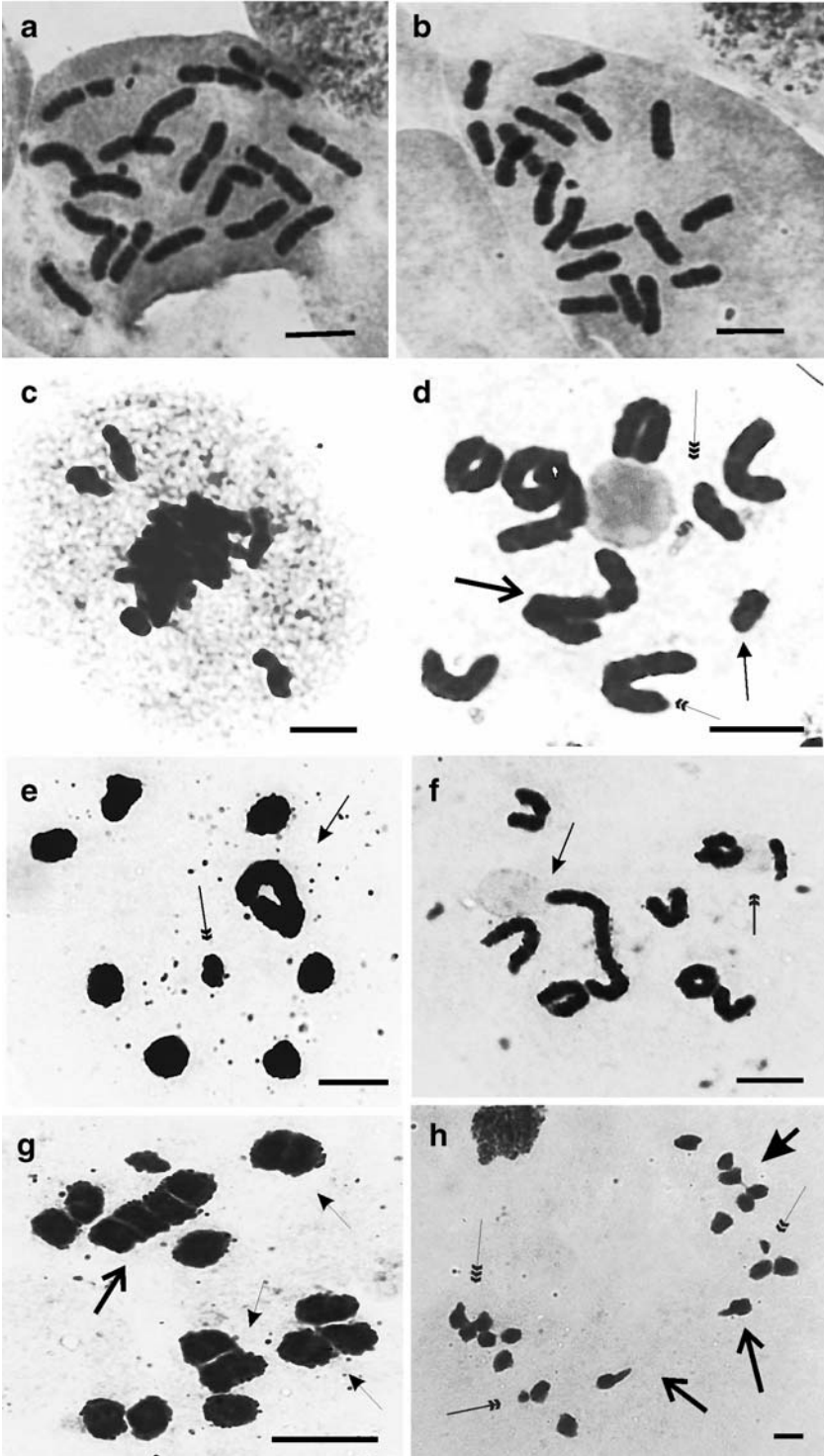
◀ **Fig. 2** Somatic metaphase and meiotic configuration of *Santolina impressa*. **a** somatic metaphase, $2n = 2x = 18$, $12m + 2m^{sat} + 2sm^{sat} + 2st$, population Nr. 6; **b** somatic metaphase, $2n = 2x = 18$, $12m + 2m^{sat} + 3sm+1sm^{sat}$, population Nr. 5; **c** prophase I, three unoriented chromosomes, population Nr. 6; **d** diakinesis, $2n = 2x = 18 = 1\ I + 4\ CII + 1\ CII - 1\ frag + 2\ OII + 1\ CIII + 1\ frag$; the thick arrow indicates trivalent chains; the thin and double arrows indicate a chromosome fragment due to breakage in the chromosome arm of the open bivalent; the triple arrow indicates a univalent; two bivalents associated with the nucleolus are observed, population Nr. 6; **e** diakinesis, $2n = 2x = 18 = 1\ I + 1\ CII + 6\ OII + 1\ OIII$; the arrow indicates a quadrivalent ring, the double arrow indicates a univalent, population Nr. 3. **f** diakinesis, $2n = 2x = 18 + 1B = 5\ CII + 2\ OII + 1\ CIV + 1\ BII$; the single arrow indicates an open bivalent and one chromosome of the quadrivalent chain associated with the nucleolus; the double arrow indicates a bivalent ring and a B bivalent associated with the nucleolus, population Nr. 3; **g** abnormal anaphase I; the thick and thin arrows indicate delayed disjunction of the four non-homologous and the two homologous chromosomes respectively, population Nr. 3; **h** anaphase I; thick arrows indicate chromatin bridges without fragment; double arrows indicate B chromosomes showing normal segregation; the triple arrow indicates the delayed disjunction of the three non-homologous chromosomes; the arrow head indicates interchromosomal adhesion or delayed disjunction of homologous and non-homologous chromosomes, population Nr. 6. Scale bar – 6 μm. For population origin see Table 1

fruiting were also variable, with a range of 75–200 achenes (mean ± s.d. = 128.45 ± 57.21) in the former, and a range of 30.02–91.22% (mean ± s.d. = 58.95 ± 30.53%) in the latter. These variables did not prove to be statistically significant ($P>0.05$) for any sources of variation analyzed by means of nested MANOVA (between populations Wilk's $\lambda=0.83$, $F_{15,235.04}=1.09$; between individuals in the populations Wilk's $\lambda=0.11$, $F_{243,255.87}=1.14$).

Discussion

The base chromosome number of *Santolina impressa* is $x=9$, which agrees with that proposed for the genus *Santolina* (Valdés-Bermejo and Antúnez 1981) and for

Table 2 Nested MANOVA of chromosome morphology variation in *Santolina impressa*

Source of variation	Multivariate analysis				Univariate analysis (dfr=180)				
	Wilk's λ	F	dfe	dfr	dfe	LBC	LBL	LTC	r
						F (VCP)	F (VCP)	F (VCP)	F (VCP)
BPP	0.30	22.22***	12	468.59	3	30.37 (1.50)***	76.19 (1.30)***	123.93 (1.40)***	1.18 (0.00) n.s.
AIP	0.04	14.32***	64	695.20	16	35.91 (33.60)***	70.33 (74.60)***	125.57 (80.30)***	3.06 (1.10)**
APP	0.003	3.71***	640	710.77	160	5.64 (45.40)***	1.47 (4.60)*	2.35 (7.90)***	18.53 (89.80)***

LBC – length of the short arm of the chromosome; LBL – length of the long arm of the chromosome; LTC – total length of the chromosome; r – chromosome ratio; dfe – degree of freedom of the effect; dfr – degree of freedom of the error; BPP – between populations; AIP – between individuals in each population; APP – between chromosome pairs in each population and individual; VCP – variance components (%); n.s. – $P>0.05$; * – $P<0.05$; ** – $P<0.001$; *** – $P<0.0001$
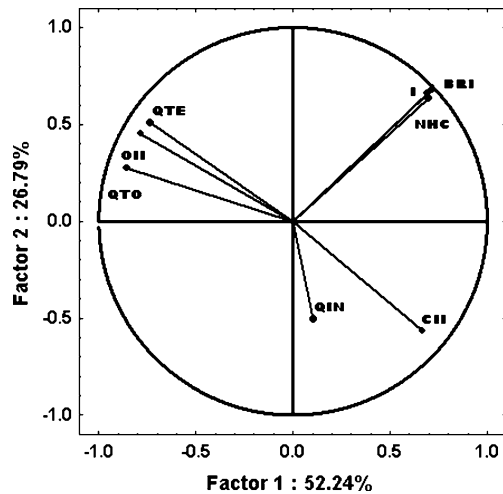
Fig. 3 Projection of the meiotic variables for the first two extracted factors of PCA. I – univalents; OII – bivalent rings; CII – open bivalents; BRI – frequency of simple chromosome bridges without fragments in anaphase I; NHC – frequency of delayed disjunction of the four non-homologous and the two homologous chromosomes; QTO – total chiasma frequency; QTE – terminal chiasma frequency; QIN – interstitial chiasma frequency

S. pectinata, *S. rosmarinifolia* subsp. *rosmarinifolia*, *S. rosmarinifolia* subsp. *arrabidensis*, and *S. ageratifolia* (Rivero-Guerra 2008a,b,c). Fernández and Queirós (1971) studied some plants of the Troia Peninsula, but did not mention the number of individuals studied, and did not find any variation in the karyotype of this species.

The mean chromosome length is higher in *S. impressa* than in other taxa of the *S. rosmarinifolia* aggregate, except for *S. semidentata* subsp. *melidensis* (mean ± s.d. = $6.14 \pm 0.13 \mu$m), but the intrachromosomal asymmetry index is greater in *S. ageratifolia* (mean ± s.d. = 0.35 ± 0.03) (Rivero-Guerra 2008c); however, the interchromosomal asymmetry index shows similar values among taxa. In general, the karyotypes show low values of asymmetry, as is common in the tribe Anthemideae (Schweizer and Ehrendorfer 1983).

The high frequency of open bivalents is indicative of a partial failure of synapsis in the chromosome arms or is due to non-formation of crossing-over in some of the arms (Jos et al. 1968a; Ferreira 1985). The high frequency of terminal chiasmata may result either from terminalization process in the latter stages of prophase or from chiasmata localization on the distal part of the bivalent.

The variation in the number of each type of chromosome in *S. impressa* and the presence of a meiotic configuration above the bivalent level in this taxon can be attributed to chromosome translocations and/or chromosome inversions. This phenomenon also occurs in *S. pectinata* (Rivero-Guerra 2008a) and in *S. ageratifolia* (Rivero-Guerra 2008c). The presence/absence of sub-telocentric chromosomes suggests Robertsonian translocations.

The presence of chromosome bridges in anaphase I is indicative of *i*) heterozygous chromosome inversions (Sybenga 1975; Stein et al. 2004), *ii*) pairing between chromosomes that are not completely homologous, so that there is a delay in

disjunction (Raina and Khoshoo 1971), *iii*) delayed separation of two bivalents, perhaps due to the arrest of terminalization of chiasmata (Jos et al. 1968a), or *iv*) breakage and reunion processes in prophase (Gustafsson 1972). The first two hypotheses may explain the presence of chromosome bridges in anaphase I in this taxon. Furthermore, the presence of multivalent configurations in diakinesis and chromosome bridges in anaphase I suggest a hybrid origin for *S. impressa*, but the morphological variation within and between populations does not support it (Rivero-Guerra, unpubl. data).

B chromosomes are paired in diakinesis, and show normal segregation at anaphase I. They may arise as a consequence of the translocation or inversion in the chromosomes that participate in multivalent configurations, or from the fusion of some fragments, and/or from centromeric fragments (Jackson 1965; Jones and Houben 2003; Palestis et al. 2004), or by deletion of chromosomes arms (Jones 1991). They show non-chiasmata association with the standard complement.

The presence of univalents presumably indicates the failure of pairing at pachytene (Sisodia 1971; Jackson 1991; Spies et al. 1992; Belay and Merker 1997), or may result from the precocious separation of chromosomes at an early or late stage of meiotic division (Jos et al. 1968b), or from competitive chromosome pairing (supporting the presence of trivalents in diakinesis), or be a consequence of inadequate homology for chromosome pairing. If these univalents do not attach to the spindle, they may persist as laggards or become excluded from the nucleus.

The pollen stainability is higher in *S. impressa* than in *S. rosmarinifolia* subsp. *arrabidensis* (mean ± s.d. = 65.72 ± 12.51%), in tetraploid cytotypes of *S. pectinata* (mean ± s.d. = 51.95 ± 11.81%), and in *S. ageratifolia* (mean ± s.d. = 40.56 ± 25.67%), but lower than in diploid cytotypes of *S. pectinata* (mean ± s.d. = 92.69 ± 15.23%); however, pollen stainability of *S. rosmarinifolia* subsp. *rosmarinifolia* (mean ± s.d. = 94.75 ± 13.45%) is still higher. The plants of *S. impressa* with chromosome rearrangement manifest considerable sterility, amounting to nearly 50 per cent. The deleterious effect of univalents on pollen stainability observed in *S. impressa* has been documented by Simonsen (1972) in *Phleum pratense*, and by Fiona et al. (1988) in *Solanum muricatum*. However, Dnyansagar and Sudhakaran (1970) in *Vinca rosea*, Merker (1971) in Triticale, Gatt et al. (2000) in some hybrids of *Dahlia*, and Palma-Silva et al. (2004) in *Vriesea* and *Aechmea* demonstrated that the frequency of univalents is not correlated with pollen stainability. Furthermore, the deleterious effect of chromosome bridges in anaphase I on pollen stainability observed in *S. impressa* has been documented by Gustafsson (1972) in *Atriplex longipes*, by Sisodia (1971) in *Urochloa*, by Spies et al. (1992) in *Tribolium*, Lu (1993) in *Elymus*, by Atlagić (1996) in *Helianthus*, Costa and Forni-Martins (2004) in *Echinodorus tennellus*.

Pollen sterility observed in some plants of *S. impressa* indicates single crossing-over within the inverted segment in most of the cells, giving rise to nearly 50% duplication-deficiency gametes. In spite of the deleterious effect of the abnormal meiotic chromosome behaviour on pollen stainability, the mean pollen stainability is high for the remaining individuals in the populations. In these cases, there are two possibilities: either that the frequency of the inversions in the populations is low, or that the crossing-over frequency within the inverted segment is low, and consequently a low number of duplication-deficiency gametes has been formed. In

addition, the low frequency of interstitial chiasmata, a seemingly low frequency of inversions, and a high frequency of normal anaphase I suggest that the inverted segments are localized mostly in the interstitial region. The same occurs in *S. semidentata* subsp. *semidentata*, *S. semidentata* subsp. *melidensis*, *S. canescens,* and in the hybrid complex of *S. rosmarinifolia* subsp. *rosmarinifolia*, *S. oblongifolia* and their putative hybrids (Rivero-Guerra 2009).

However, in diploid and tetraploid cytotypes of *S. pectinata* (Rivero-Guerra 2008a) and in *S. ageratifolia* (Rivero-Guerra 2008c), the frequency of interstitial chiasmata (mean ± s.d. = 7.28 ± 1.31, 18.33 ± 3.54 and 27.97 ± 5.99, respectively) is higher than the frequency of terminal chiasmata (mean ± s.d. = 4.95 ± 1.62, 10.38 ± 4.88 and 14.03 ± 6.62, respectively); the contrary occurs in the remaining taxa of the *S. rosmarinifolia* aggregate. This indicates that the crossing-over frequency within the inverted segment is high, and consequently the number of duplication-deficiency gametes is high. This explains the mean value of pollen stainability in the tetraploid cytotype of *S. pectinata* (Rivero-Guerra 2008a) and in *S. ageratifolia* (Rivero-Guerra 2008c). It also explains the lower percentage of fruiting of these two taxa (49.91% in the tetraploid cytotype of *S. pectinata* and 34.24% in *S. ageratifolia*) than in the other taxa of this aggregate.

In general, the fecundity is higher in *S. impressa* than in *S. rosmarinifolia* subsp. *arrabidensis* (Rivero-Guerra 2008b), in *S. ageratifolia* (Rivero-Guerra 2008c), and in diploid and tetraploid cytotypes of *S. pectinata*, but lower than that found in *S. rosmarinifolia* subsp. *rosmarinifolia* (Rivero-Guerra 2008b). The results show that the karyotype morphology variation and meiotic irregularities have no significant effect on fecundity; the same occurs in *Haworthia reinwardtii* var. *chalumnensis* (Brandham 1974).

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Appendix 1. Studies specimens of *Santolina impressa*

Portugal: Setúbal: Nos Montes, May 1900, *Luisier 7402* (Inst. Nun' Alvres, Caldas de Saudí); carretera de Alcácer do Sal a Troia, cruce hacia Batalha, 38°23′42″ N 8°34′35″ W, 19 m, 26 May 1998, *Rivero-Guerra 331* (ARG), *242706* (SEV); idem, a 0.2 km del cruce a MonteJunl, 38°23′55″ N 8°36′54″ W, 13 m, 26 May 1998, *Rivero-Guerra 332* (ARG), *242705* (SEV); idem, Comporta, 38°22′46″ N 8°46′28″ W, 14 m, 26 May 1998, *Rivero-Guerra 333* (ARG), *242707* (SEV); idem, km 13, 38°23′45″ N 8°44′00″ W, 18 Aug 1998, *Rivero-Guerra 450* (ARG); idem, km 26, 38º23′19″ N 8º45′48″ W, 18 Aug 1998, *Rivero-Guerra 451* (ARG); idem, km 36, 38º23′40″ N 8º47′03″ W, 18 Aug 1998, *Rivero-Guerra 452* (ARG); Península de Troia, 29 May 1972, *Rivas Goday & Rivas Martínez 82971* (MAF); idem, 1 Jun 1978, *Barbosa 119717* (SEV), *411653* (MA); idem, 10 m, 26 Jun 1961, *M. da Silva* 2290 *59107* (LISE); idem, entre as instelacoes de OTAN e as dos Fuzileiros, 4 Jul 1940, *Nedros* (ISAL); idem, pr. ao Hotel Bico das Lulas, 25 Sept 1972, *G. Costa 23976* (PO); idem, Caldeira de Troia, 18 Jul 1983, *Gómez Pedro & J. F. Coste 24082* (ISAL); idem, próx. da Mallia da Costa, 27 Jun 1971, *Malato-Beliz 11225 & al. 246888* (MA); idem, 10 Jun 1941, *Luisier 7401* (Inst. Nun' Alvres, Caldas de Saudí); idem, Troia, 38°26′08″ N 8°49′51″ W, 8 m, 18 Aug 1998, *Rivero-Guerra 453* (ARG), *242708* (SEV); carretera de Troia a Sines, Torre, 38°20′35″ N 8°46′27″ W 24 m, 18 Aug 1998, *Rivero-Guerra 454* (ARG), *242709* (SEV); idem, en el cruce Grándola-Sines, dirección Sines, 38°00′05″ N 8°49′46″ W, 34 m, 18 Aug 1998, *Rivero-Guerra 455* (ARG), *242710* (SEV); idem, 8 km pasado Pineiro da Cruz dirección Sines, 38°14′59″ N 8°45′02″ W, 18 Aug 1998, *Rivero-Guerra 456* (ARG); Grándola, praia de Santa Maria, 31 May 1968, *Rozeira & A. Serra 23973* (PO); idem, entre Sto. André e Melides, 29 May 1968, *Rozeira & A. Serra 23972* (PO); idem, Lagoa de Melides, 29 May 1968, *Rozeira & A. Serra 23971* (PO); idem, Melides; Herdade do Chapanel, 2 Oct 1990, *J. C. Costa & Espírito Santo* (ISAL); idem, entre a lagoa eo mar, 17 Mar 1990, *Espírito Santo* (ISAL); idem, Torre, praia, 17 May 1977, *Gómez Pedro 22162* (ISAL); idem, Cawalteal, entre a praia e a fovoacao, a Malhade Alta, 7 May 1977, *Gómez Pedro 22180* (ISAL); idem, Pinheiro da Cruz, praia de Pinheiro da Cruz, 19 Jul 1983, *Gómez Pedro & J. F. Costa 24089* (ISAL); San Lorenzo de Azeitao, Alto da Madalena, 12 May 1987, *Gómez Pedro 24989* (ISAL); Alcácer do Sal, Comporta, cerca de 3 km

desta povoaçao na estrada para Alcácer, 19 May 1990, *J. C. Costa & Lousa* (ISAL); Entre Alcácer do Sal y Comporta, 30 m, 19 May 1990, *J. Amigo 020302* (SANT); Santiago do Cacém, entre a Lagoa de Melides e a Lagoa de Santo André, 30 Jun 1978, *Barbosa 12972* (ISAL); Sines, terrenos arenosos, 5 Jul 1951 *Rainha 2175, 08620* (SANT); idem, pr estacao de caminho de ferro, 50 m, 17 May 1962, *Rainha 5475, 71296* (LISE); idem, a W, Parque Municipal de Turismo, 29 May 1970, *Gómez Pedro 182* (ISAL); idem, a W do Farol, 28 May 1970 *Gómez Pedro* (ISAL); idem, a la salida del pueblo dirección Santiago do Cacém, 26 May 1998, *Rivero-Guerra 330* (ARG); ibidem, 18 Aug 1998, *Rivero-Guerra 449* (ARG); Cabo de Sines, 24 May 1979, *Castroviejo & Valdés-Bermejo 5201, 445938* (MA); idem, 28 Jun 1956, *Malato-Beliz 3295 & al. 246889* (MA); idem, 15 Jun 1971, *de Vasconcellos* (ISAL); idem, Sep 1940, *de Vasconcellos & M. Torres* (ISAL).