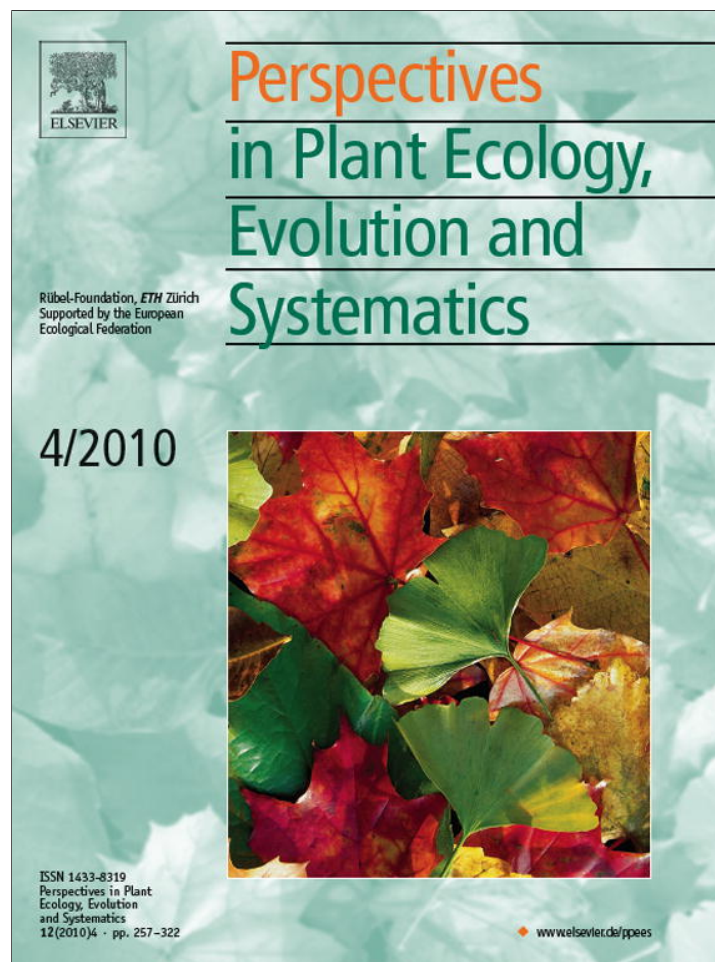


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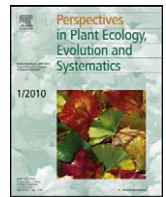
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Research article

Why are fruit traits of *Cistus ladanifer* (Cistaceae) so variable: A multi-level study across the western Mediterranean region

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ABSTRACT

Variation is the raw material for evolution. Differences among populations in the expression of traits related to plant fitness may result from natural selection, phenotypic plasticity (in response to local conditions), and developmental instability (manifested as high intra-individual variation in repeated patterns or characters). *Cistus ladanifer* is a highly polymorphic plant distributed in the Mediterranean and the only species in the family (Cistaceae, 180 species) with a variable number of valves per fruit. We herein analysed the variation in the number of valves (5–12) and seeds (318–1185) per fruit in 36 populations (607 individuals, 1821 fruits) at different levels: temporal, geographical, ecogeographical, taxonomic, and phylogeographical. In addition, we tested whether an increased number of fruit valves influences pre-dispersal seed predation. Large variation in the number of valves and seeds per fruit among populations, individuals, and years was reported. The number of ovules and seeds per valve increased with a higher number of fruit valves. Geographical and taxonomic variables did not significantly explained this fruit variation. On the contrary, we found a negative relationship between the number of fruit valves and altitude and a positive relationship with precipitation. We argue that ecogeographical factors, in addition to some phylogeographical and phylogenetic signals, are involved in the multiplication of carpels during the development of the ovary wall. This, coupled with the results of the evolutionary history of *Cistus* in previous studies, indicates active evolutionary processes in *C. ladanifer* populations.

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Introduction

“Isidore Geoffroy Saint-Hilaire insists that, when any part or organ is repeated many times in the same animal, it is particularly liable to vary both in number and structure. . . Whenever such parts as the vertebrae or teeth, . . . , or petals, stamens, pistils, or seeds, are very numerous, the number is generally variable.” – Darwin (1868, chapter XXVI, p. 342).

Flower and fruit features display little variation within taxa and have been used as essential materials in the classification of angiosperms at all taxonomic levels (Caesalpinus, 1583; Linnaeus, 1753). For instance, fruit traits are used to delimit species of the genus *Valerianella* (Coode, 1967), and the number of capsule valves is used to segregate genera in Caryophyllaceae (*Melandrium*, *Silene*, *Cerastium*) (Talavera, 1987). Similarly, little variation is expected in

key flower characteristics, as flowers are directly involved in the first stages of reproductive success. Because flower and fruit traits determine processes involved in fitness (e.g., pollination, dispersal, or pre-dispersal seed predation), they may be selected towards an optimal level and thus display little variation through strong stabilising selection; this is in contrast to vegetative traits, which affect reproductive output in an indirect manner (Armbruster et al., 1999; Sultan, 2000; Brock and Weing, 2007). Many existing studies explain the variation in reproductive traits at different levels (Totland and Birks, 1996; Méndez and Gómez, 2006); however, the individual level is rarely taken into account (Vogler et al., 1999; Guitián et al., 2003). Studying the variation within individuals could provide results of paramount importance about the stability of these traits, as well as the adaptive value of the variations (Herrera, 2009).

Cistaceae comprises eight genera and about 180 species (Arrington and Kubitzki, 2003) that have a syncarpous or eusyncarpous 3-carpellated gynoecium with numerous ovules. Mature fruits in the Cistaceae primarily have three opened parts (valves) resulting from the development of three carpels. The only excep-

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tion to this pattern is *Cistus*, which primarily develops five-valved fruits. However, *Cistus ladanifer* is the only species of *Cistus* that exhibits a variable number of carpels and fruit valves (6–12) (Demoly and Montserrat, 1993). The variability of this character is not only unique to Cistaceae, but also rare in the angiosperms as the structural parts of flowers and fruits are extremely stable within species (Takhtajan, 1981; Endress, 1994). Nandi (1998) observed that numerous flower characteristics in Cistaceae displayed limited variation, except for ovary division in the gum rock-rose (*C. ladanifer*). Thus, this species is an ideal model species to explore multiplication of fruit valves during the development of the ovary wall.

A historical reconstruction of the number of fruit valves within Cistaceae clearly showed a transition from fruits from three to five or more valves (Guzmán and Vargas, 2005). *C. ladanifer* produces a large number of flowers (2–386) and fruits (0–300) at the apex of each branch of the past year depending on particular conditions (Talavera et al., 1993), but the causes of variation in the number of valves remains elusive. Multi-level descriptions of valve number variation and its ecological and geographical correlates may shed light on these causes. Ecogeography accounts for numerous clinal patterns of plant differentiation at the species and population level, particularly when populations must cope with complex environments (Endler, 1986; Petru et al., 2006; Pérez-Barrales et al., 2009). Variation in plant traits across environmental gradients may be genetically determined (Joshi et al., 2001; Petru et al., 2006) or may be simply a plastic response (Vogler et al., 1999; Erfmeier and Bruelheide, 2009). Although phenotypic plasticity, i.e. the ability of an organism (genotype) to change its phenotype in response to changes in the environmental conditions, may involve variation in reproductive characters, most studies have focused primarily on vegetative traits, such as branches and leaves (Sultan, 2000; Herrera, 2009). Phenotypic plasticity itself may be an adaptive response, as it helps organisms to cope with variable environments (Pigliucci, 2000; Sultan, 2000; Brock and Weinig, 2007). In addition, some studies have shown that variation still exists even when controlling for both genetic and environmental causes of phenotypic variation. Variation caused by developmental instability is well known for morphological traits and can manifest, for instance, as fluctuating asymmetry (Freeman et al., 1994).

The gum rock-rose is a woody perennial shrub occurring in a wide range of habitats on both the European and the African sides of the western Mediterranean. Fruit characteristics make *C. ladanifer* especially suitable for investigation of trends in valve variation across the species distribution. A multi-level study may allow us to distinguish between the contribution of genetic/developmental constraints and environmental conditions to fruit trait variation. Additionally, increased fruit valve and seed number of *C. ladanifer* may decrease the risk of complete pre-dispersal predation (Serrano et al., 2001) given that these plants are often consumed by Noctuidae (Lepidoptera) and Bruchidae (Coleoptera) (Serrano et al., 2001; Bastida and Talavera, 2002; Delgado et al., 2007). Alternatively, it is possible that variation in valve number only reflects co-ancestry with other populations that already showed this variation, leading to a geographic pattern more related to historical processes than to contemporary selective pressures.

The aim of the present work was to assess the variability in the number of valves and seeds per fruit in *C. ladanifer* and investigate whether this variability is related to ecogeographical factors, genetic factors, or seed predation pressure. Specifically, we addressed (1) whether there is significant variation in the number of valves and seeds per fruit within and among individuals and populations and, if so, whether this variation is constant through time; (2) the trade-offs between the production of ovules and seeds per valve and the number of valves per fruit; (3) the ecogeographical conditions such as precipitation, temperature, or altitude that may

be involved in fruit variation; (4) the relationship between the pattern of seed predation and the number of valves per fruit; and (5) the extent to which fruit variation is correlated with taxonomy and phylogeography.

Methods

Study species

C. ladanifer L. is a western Mediterranean shrubby species occurring in a wide range of latitudes (33–43°), altitudes (0–1500 m), and climates (dry to humid) with a geographical distribution in southern France, Portugal, and Spain, as well as northern Morocco and northern Algeria (Demoly and Montserrat, 1993) (Fig. 1A). This insect-pollinated species colonises degraded areas, and its abundance has increased in the past few decades due to human disturbance (Trabaud, 1995; Luis-Calabuig et al., 1996).

Morphological variation in the vegetative characteristics of *C. ladanifer* differs for three subspecies: *ladanifer*, distributed in Spain, Portugal, France and northern Morocco; *africanus*, distributed in southern Spain (Cádiz and Málaga provinces), northern Morocco and northern Algeria; and *sulcatus*, endemic to southwest Portugal (Algarve region) (Demoly and Montserrat, 1993).

The fruits of *C. ladanifer* are globular lignified capsules with 6–12 valves (Fig. 2). The plant produces a large number of seeds that remain inside the capsules until summer, when fruit valves open and seeds fall near the mother plant (Bastida and Talavera, 2002). The gum rock-rose is an obligatory-seeder plant. The population explosively regenerates through the soil seed bank after an intense disturbance such as fire (Valbuena et al., 1992; Delgado et al., 2001).

This study was conducted from 2003 to 2008 in 36 populations throughout the species distribution area (Appendix A and Fig. 1B–D). All study locations were natural patches where *C. ladanifer* was the dominant species.

Levels of variation

We explored whether the number of valves and seeds per fruit varied among and within individuals and populations. In 2005, two to four fruits each were randomly collected from 6 to 21 individuals in each of 34 populations (Appendix B). The variability in the number of seeds per fruit was analysed in a subset of 19 populations in which all fruits were mature but unopened (Appendix B). We estimated the number of seeds per fruit by counting two opposite valves (one to three fruits in 4–16 individuals, Appendix B) and multiplying the mean number by the number of valves. Fruits with external signs of seed predation were not used for this estimate. Other possible sources of variation in fruit traits were explored in subsets of these populations:

1. *Temporal variation.* Fruits of 6–20 marked individuals from 19 Iberian populations (Appendix B) were analysed in 2004 and 2005. For each individual, four fruits on branches facing the four geographical points were randomly collected and the number of valves was counted. A subset of six populations was selected for the same study performed in 2006 (Appendix B).
2. *Geographical variation.* We explored the existence of a geographical pattern (latitude and longitude) in the variation of the number of fruit valves and seeds per fruit in 34 and 19 populations, respectively (Appendices A and B).
3. *Ecogeographical approach.* We considered altitude, mean annual temperature, mean maximum temperature of the hottest month, and mean annual precipitation (Appendices A and B).
4. *Taxonomy.* We compared the number of (1) valves and (2) seeds in populations of two widespread *C. ladanifer* subspecies: subsp.

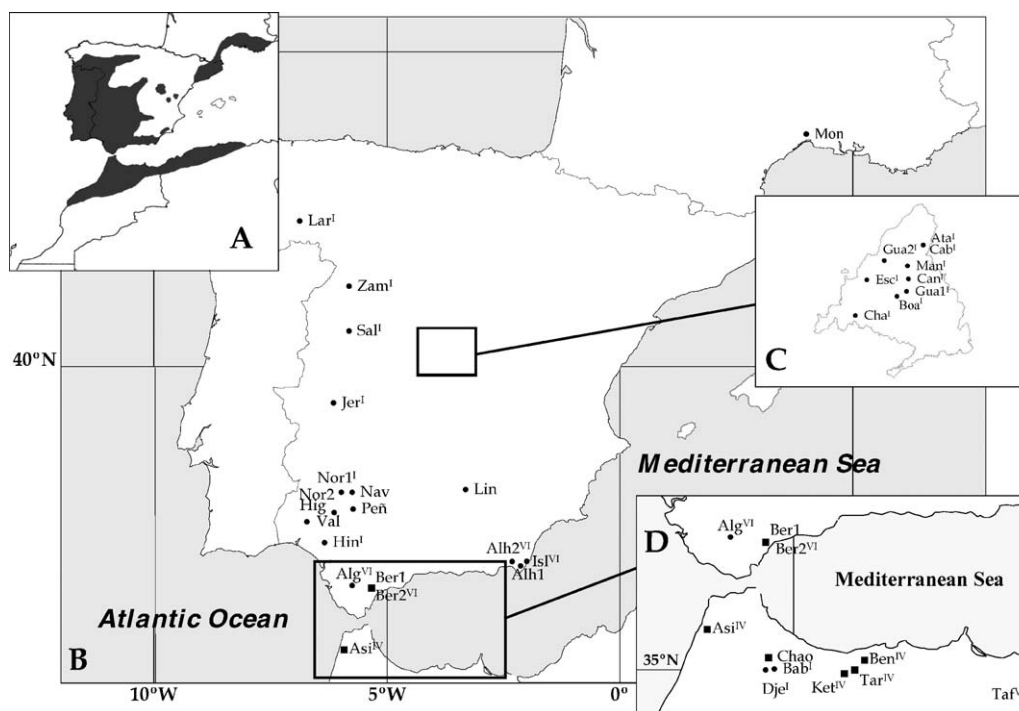


Fig. 1. Distribution area (A) and geographical location of 36 studied populations of *C. ladanifer* from Spain, France and northern Morocco (B). Detailed map of the Madrid province (C) and the Strait of Gibraltar region (D). Population abbreviations as in Appendix A. Each full circle (●) represents one population of *C. ladanifer* subsp. *ladanifer*, and a full square (■) represents one population of *C. ladanifer* subsp. *africanus*. Roman numerals after population abbreviations indicate haplotypes following Guzmán and Vargas (2009) and this study.

ladanifer ($n_1 = 27$, $n_2 = 16$) and subsp. *africanus* ($n_1 = 7$, $n_2 = 3$) (Appendices A and B).

- Phylogeography.** We analysed the relationship between the variation in the number of fruit valves and the haplotypes found in *C. ladanifer* (Guzmán and Vargas, 2009). The sequencing of the *trnS-G* plastid and *trnK-matK* spacers revealed four haplotypes in 26 populations of the present study (Appendices A and B, Fig. 1). For the valve analysis, the Moroccan population of Taforalt ("Taf") was not included in the analysis because it was the only population with the rare haplotype 7. However, we did not analyse the relationship between haplotypes and number of seeds per fruit because all populations showed the same haplotype (number one), except for "Ber" with haplotype 6 and "Ket" with haplotype 4; this violated replicate requirements.
- Trade-off.** To determine whether there were compensatory effects (trade-offs) between the production of ovules and seeds per valve and the number of valves in each fruit, we counted the number of seeds and aborted ovules in two opposite valves of three to five fruits in 20 individuals of four populations (Appendix B). The possible relationship between the number of fruit valves and the total fruits produced per individual was assessed in 9–32 plants of four populations (Appendix B); in two populations (Nor1 and Nor2), the analysis was performed in two successive years (2004, 2005). For each individual, we counted the total number of fruits and the number of fruit valves in four fruits.
- Fruit and seed predation.** We investigated whether pre-dispersal seed predation is affected by the number of total fruit valves at both the inter-population and the intra-population levels. To analyse the overall range of variation in seed predation, 20 fruits from 20 plants of six populations were analysed in 2008 (Appendix B). We selected three populations with a high number of valves and three populations with a low number (see section 'Results'). Fruits were collected in the first week of September

after they had completely matured and the period of insect predation had finished (Serrano et al., 2001). The number of valves of each fruit and the number of valves affected by seed predators was then counted. Fruits were affected by only one larva because only one exit hole was found; rare cases of fruit with two or three holes were found in Boa and Alh2 populations. To collect seed predators, some predated fruits of each population were left in paper bags; two months later, bags were re-examined and insects in their adult stage were found and identified. We also explored whether pre-dispersal seed predation was related to haplotype or environmental factors (Appendix A).

Statistical analyses

Overall variation in the number of fruit valves was explored with generalised linear models (GLM) with a log link function and a quasi-Poisson error structure (Dobson, 2001; Crawley, 2005), where "population" and "plant" (nested in "population") were the main factors. We utilised quasi-functions and F tests to correct for underdispersion (Crawley, 2005). Deviance quotients provided the percentage of relative variance accounted for by each level, where the error deviance represented within-individual variance (García et al., 2005). Variation in the number of seeds per fruit was analysed using a nested ANOVA in which the factor "population" was random and the factor "plant" was random and nested within "population." Data were logarithm transformed.

To test whether the number of fruit valves varied among years, we performed a generalised estimating equations (GEE) analysis, which is an extension of the GLM that allows analysis of repeated measurements on the same subject (Dobson, 2001). Data from two and three consecutive years were analysed separately due to the different number of populations sampled. The mean number of valves for each plant was treated as the dependent variable, and "plant" was nested within "populations." Gaussian distribution of

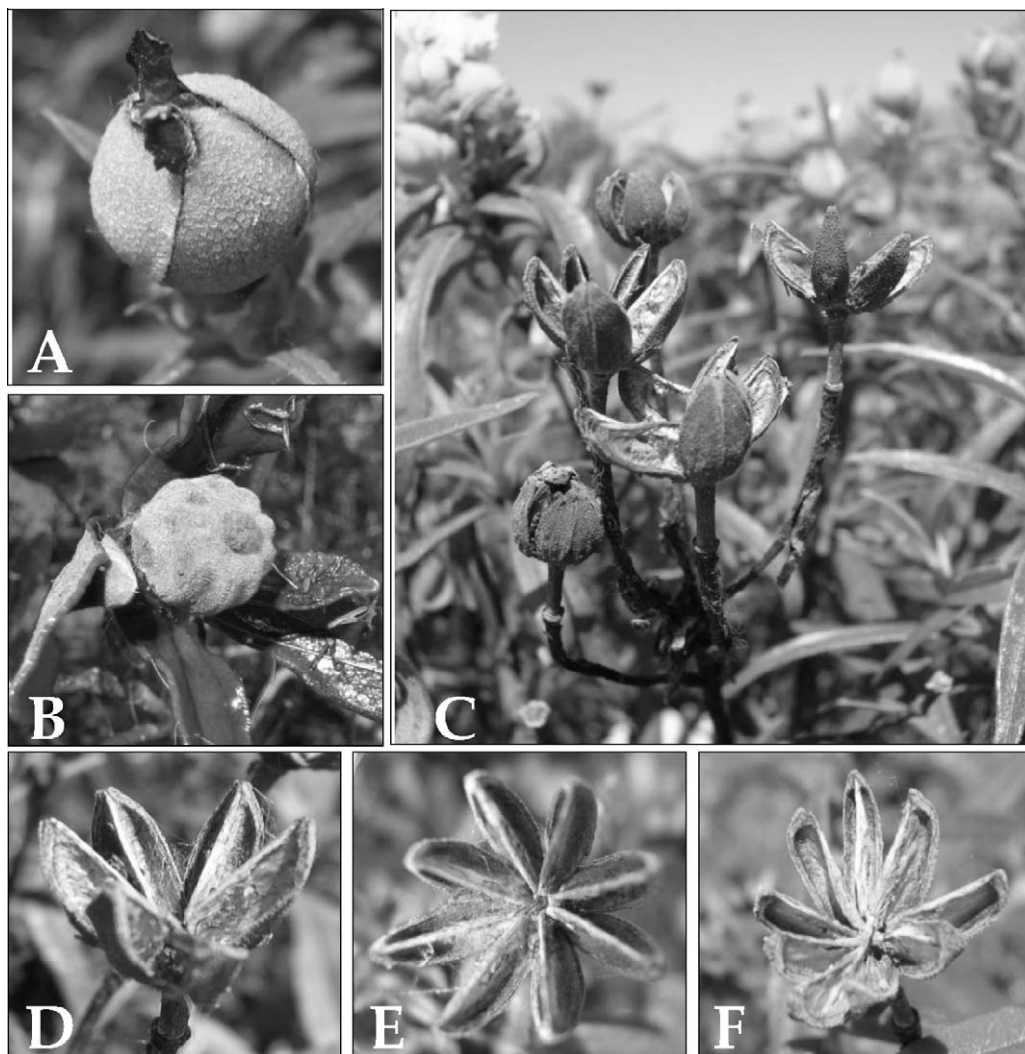


Fig. 2. *Cistus ladanifer* fruits. (A) Immature capsule covered by the sepals. (B) Mature capsule with falling sepals. (C) Inflorescence of mature and opened capsules at the end of woody pedicels. Opened capsules with six (D), eight (E), and nine valves (F). All photographs by Beatriz Guzmán.

errors with a log link function was used because it fits the observed data best.

Spatial statistics were used to explore the geographical component of variation in the number of valves and seeds per fruit among populations. The spatial autocorrelation in the two variables was explored by means of semivariograms in which the proportion of total variance explained by spatial dependence was expressed by the structural fraction, and the fit of the model to the semivariance analysis was assessed using the model R^2 . To test the relationship between the number of valves and seeds per fruit and four ecogeographical variables (altitude, T , T_h , PREC; Appendix A), a multiple regression analysis was carried out.

Variations in the number of valves between subspecies were tested with a GLM, assuming a Poisson error distribution and a log link function, in which the factor “population” was nested within “subspecies” and “plant” was nested within “populations.” Differences in the number of seeds per fruit between subspecies were analysed using a mixed-model ANOVA, in which the factor “subspecies” was fixed and the factor “population” was random and nested within subspecies, and the data were logarithm transformed. A GLM was similarly performed to determine differences in the number of valves per fruit between haplotypes; in this GLM, the factor “population” was nested within “subspecies” and “plant”

was nested within “populations.” Additionally, to test whether populations with identical haplotypes have similar numbers of fruit valves, a Nei unbiased genetic distance matrix based on lineages obtained by Guzmán and Vargas (2009) was calculated for all pairs of populations (Appendix A) with haplotypes 1, 4 and 6. Similarly, for the number of valves per fruit, a differentiation index was computed for all pairs of populations as the absolute value of the difference between mean numbers of valves per fruit for each population pair. Correlations between both symmetrical matrices were calculated using the Mantel permutation test (Mantel, 1967), with the permutation number set to 1000.

One-way ANOVAs were also performed to explore the existence of a compensatory effect between ovules or seeds and the number of valves by comparing the number of ovules or seeds per valve in fruits with different numbers of valves. Spearman rank correlations were performed to analyse possible relationships between the number of fruit valves and the total fruits produced per individual.

To compare the number of fruit valves affected and not affected by seed predators, we used a GLM with a Poisson error distribution and a log link function. The percentage of individuals with at least one fruit predated for the populations with a low and high number of fruit valves was compared using the Mann–Whitney test. The same analysis was used to compare the percentage of fruits affected

for the populations with a low and high number of fruit valves. The percentage of predated valves per fruit among fruits with different numbers of fruit valves was compared by means of GLMs assuming a gamma error distribution and a log link function, in which the factor “plant” was nested within “populations.” The same procedure was used to analyse the percentage of predated valves in infested fruits between populations with low and high numbers of fruit valves, but with consideration of a binomial error distribution with a probit link function; the factor “population” was nested within “fruit valve production” and “plant” was nested within “populations.” Spearman rank correlations were performed to determine if there was a relationship between pre-dispersal seed predation and altitude and climatic factors. To compare pre-dispersal seed predation between the two haplotypes (1 and 6), GLMs assuming a gamma error distribution and a log link function were used.

The GLM for analysing the overall variation in the number of fruit valves was generated with R version 2.5.0 (R Development Core Team, 2007). All other GLMs, GEEs, and ANOVAs were carried out using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). The spatial autocorrelation analysis was performed with GS+ 5.0 software (Gamma Design Software, Plainville, MI, USA). GenAlEx 6 software (Peakall and Smouse, 2006) was utilised to perform the Mantel test.

Prior to conducting ANOVAs, the normality of variables was checked with the Kolmogorov–Smirnov test and homoscedasticity was tested with Levene’s test (Day and Quinn, 1989). Type III sum of squares was used for all ANOVAs because it is recommended when data are highly unbalanced (Ayres and Thomas, 1990). When ANOVAs showed significant differences, the means of groups were compared using post hoc Tukey HSD tests because the variance between groups was homogenous and unplanned comparisons were made (Day and Quinn, 1989). A hybrid of Fisher scoring and the Newton–Raphson algorithm was used for the GLMs. To control for type I error produced by multiple comparisons, we applied the sequential Bonferroni test to adjust the significance level (Rice, 1989).

Results

Overall variation

The number of valves per fruit was highly variable in the 34 studied populations in the same year, ranging from 5 (populations Alh1, Alh2, Gua1, Gua2) to 12 (Bab). The mean number of fruit valves in a population ranged from 7.2 ± 0.04 (Alh1) to 10.0 ± 0.00 (Chao, Peñ)

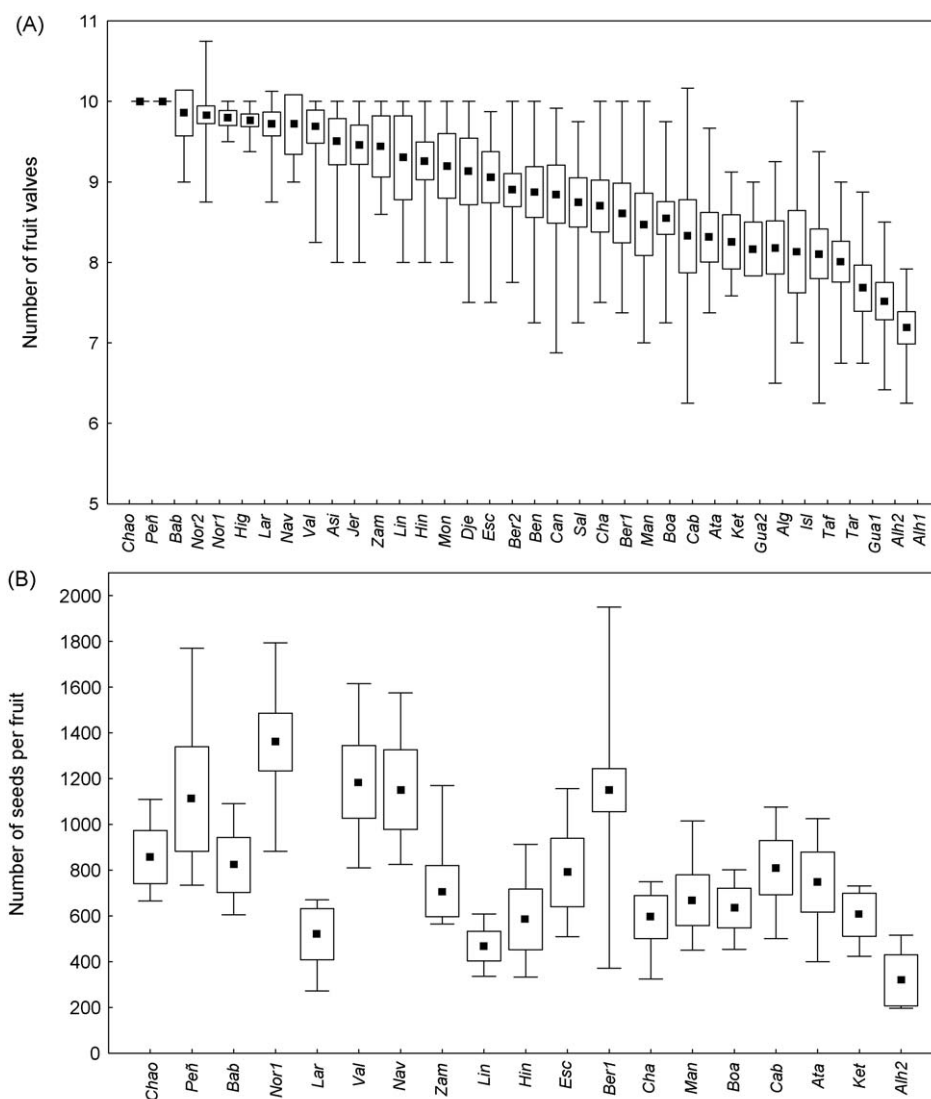


Fig. 3. Number of fruit valves (A) and seeds (B) per fruit in populations of *C. ladanifer*. The values are means (points) ± 1.96 s.e. (boxes), and minima and maxima (whiskers). See Appendix A for population codes.

Table 1

Results of the generalised estimation equations for repeated measures examining the factors affecting the number of fruit valves of *C. ladanifer* across individuals, populations and years.

	Source of variation	df	Wald χ^2	P
Two years	Year	1	21.57	<0.0001
	Population	17	1.37×10^7	<0.0001
	Plant (population)	5	3537.63	<0.0001
	Year \times Population	17	59.59	<0.0001
Three years	Year	2	27.84	<0.0001
	Population	5	1.05×10^5	<0.0001
	Plant (population)	5	17,861.66	<0.0001
	Year \times Population	10	194.24	<0.0001

(Fig. 3A). The number of fruit valves varied not only between populations and between plants of the same population, but also within the same plant. The mean number of fruit valves differed significantly among populations (GLM: $F_{33,1641} = 37.98$, $P < 0.0001$ after Bonferroni adjustment) and among individuals within populations (GLM: $F_{34,1641} = 2.09$, $P < 0.001$ after Bonferroni adjustment). Most of the fruit-valve variance was explained at the inter-population level (57.8%), although a great amount of variation was detected at the intra-individual level (40.0%), and only 2.2% of the total variance was explained by inter-individual differences. The mean population number of seeds per fruit ranged from 318.7 ± 55.83 (Alh2) to 1364.3 ± 63.14 (Nor1) (Fig. 3B). Again, the number of seeds per fruit varied significantly among populations (ANOVA: $F_{18,103} = 14.36$, $P < 0.0001$ after Bonferroni adjustment) and among individuals within populations (ANOVA: $F_{83,103} = 2.07$, $P < 0.0001$ after Bonferroni adjustment). The inter-population level accounted for the highest percentage of valve seed variance (73.9%), whereas the inter-individual and intra-individual levels explained only 14.9% and 11.2%, respectively.

Temporal variation

Repeated measures analysis showed that inter-individual and inter-population production of valves per fruit was statistically different in both the two and the three year analyses (Table 1). Moreover, temporal variation was not homogeneous in all populations. The mean number of fruit valves was stable over time for some populations, whereas it varied in others (i.e., year \times population interaction was significant, Table 1). Individuals of some populations showed a similar mean number of fruit valves across years, but in other populations, there was a large variation (Fig. 4). Additionally, we observed variation in the number of fruit valves on the same plant branch across years in some populations (Alh1, Alh2, Can, Nor1).

Geographical variation

The semivariance analyses showed that the variables poorly fit the theoretical semivariogram models. The fit for the log number of seeds per fruit was poor (Gaussian model; spatial structure = 0.86; $R^2 = 0.17$). In the case of the number of fruit valves, the fit to the theoretical model was better (spherical model; spatial structure = 0.72; $R^2 = 0.40$), but not sufficient to indicate a significant spatial structure.

Ecogeographical approach

The multiple regression of the number of fruit valves and the ecogeographical variables was significant with moderate predictive power (adjusted $R^2 = 0.45$). Altitude and mean annual precipitation explained 48% of the total variance (regression func-

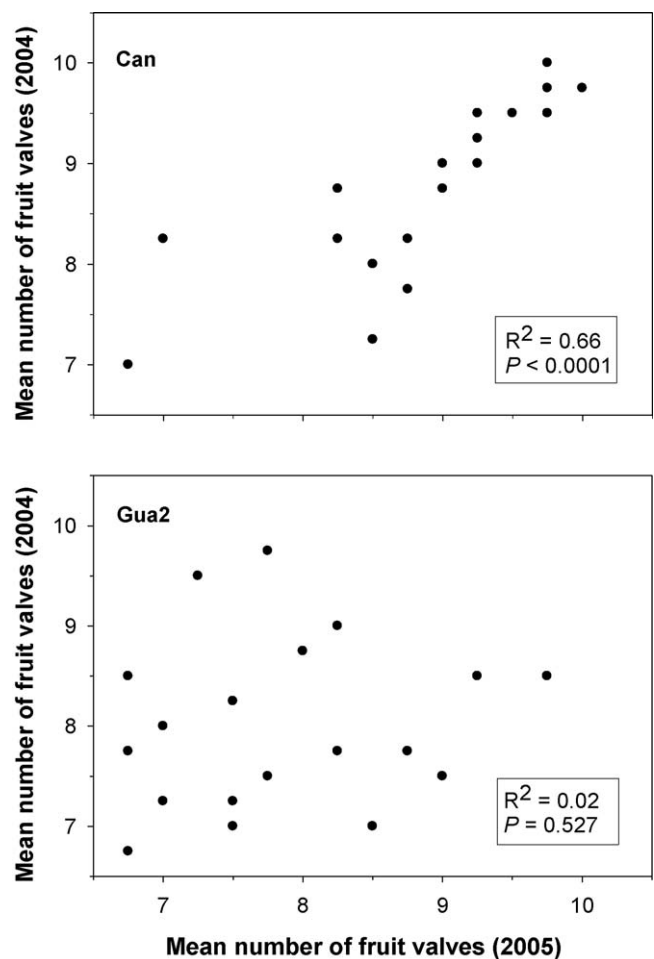


Fig. 4. Relationship between the mean number of fruit valves per plant in 2004 and 2005 in Can and Gua2 populations. Both populations were selected because they represent extreme values of R^2 . See Appendix A for population codes.

tion: number of fruit valves = $-0.51 \times \text{altitude} + 0.52 \times \text{PREC} + 8.78$; $R^2 = 0.48$; $F = 13.52$; $P = 0.00007$) (Fig. 5). However, the multiple regression of the seeds per fruit was not significant (regression function: number of seeds per fruit = $-0.39 \times \text{altitude} - 0.63 \times T + 0.42 \times T_h - 0.29 \times \text{PREC} + 4.39$; $R^2 = 0.15$; $F = 0.62$; $P = 0.65$).

Taxonomic and phylogeographical variation

The two analysed subspecies of *C. ladanifer* produced fruits with a similar number of valves (8.7 ± 0.05 for subsp. *ladanifer*, 8.8 ± 0.08 for subsp. *africanus*) and seeds per fruit (833.12 ± 29.27 for subsp. *ladanifer*, 1018.43 ± 44.72 for subsp. *africanus*; Table 2). In contrast, the mean number of fruit valves was 7.6 ± 0.27 in haplotype 6 populations, 8.0 ± 0.31 in haplotype 4 populations, and 8.7 ± 0.30 in haplotype 1 populations, and these differences were significant (Table 2). The Mantel test applied to the matrix of Nei's unbiased genetic distances and the matrix of the number of fruit valve distances for 25 *C. ladanifer* populations showed that both matrices were significantly correlated, although the relationship was fairly weak ($R^2 = 0.063$, $P = 0.021$).

Trade-off

Fruits produced a similar number of ovules per valve independently of the number of fruit valves ($F_{5,90} = 0.20$, $P = 0.96$ for Cab;

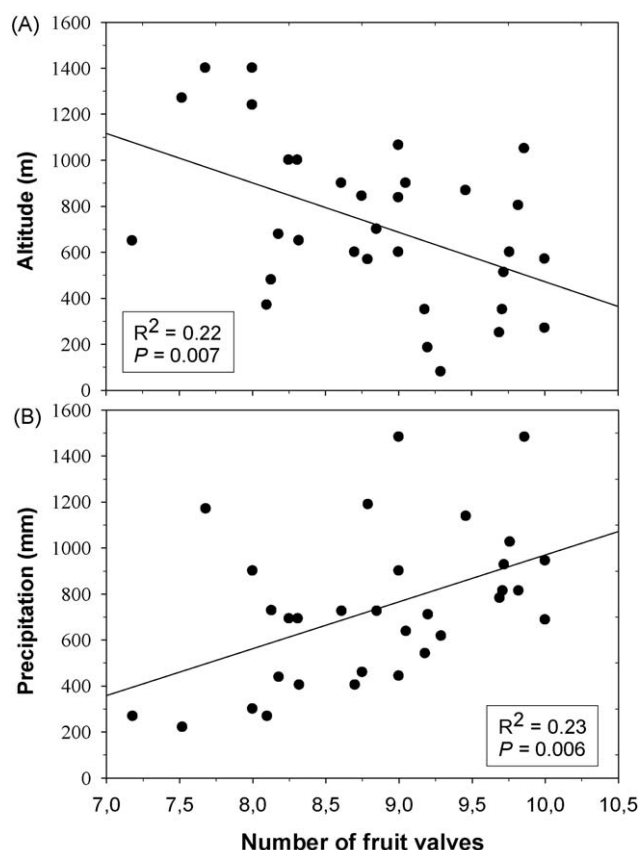


Fig. 5. Regression analysis of the number of fruit valves (mean values per population) and two ecogeographical variables: altitude (A) and mean annual precipitation (B).

$F_{4,94} = 0.48$, $P = 0.75$ for Esc; $F_{3,90} = 1.38$, $P = 0.25$ for Nor1), except for Ber1, where differences were significant ($F_{3,106} = 6.06$, $P = 0.007$). Post hoc testing revealed that the number of ovules per valve was higher for fruits with eight valves than for the rest of the fruits (7, 9 and 10 valves). A compensatory effect between the number of seeds per valve and the number of fruit valves was not found, since significant differences were not found for any population ($F_{5,90} = 0.38$, $P = 0.85$ for Cab; $F_{4,94} = 0.85$, $P = 0.94$ for Esc; $F_{3,106} = 2.02$, $P = 0.11$ for Ber1; $F_{3,90} = 0.28$, $P = 0.83$ for Nor1).

The total number of fruits produced per plant was not correlated with the number of fruit valves ($\rho = 0.28$, $N = 32$, $P = 0.12$ for Nor1 in 2005; $\rho = -0.04$, $N = 29$, $P = 0.85$ for Nor1 in 2006; $\rho = -0.25$, $N = 20$, $P = 0.29$ for Nor2 in 2005; $\rho = 0.38$, $N = 10$, $P = 0.28$ for Nor2 in 2006; $\rho = -0.11$, $N = 9$, $P = 0.78$ for Hin; $\rho = -0.05$, $N = 12$, $P = 0.87$ for Ber1; Bonferroni-corrected α for 6 comparisons = 0.0083).

Table 2

Results of a nested analysis of deviance/variance to investigate the effects of subspecies and haplotypes on the number of fruit valves and the number of seeds per fruit in populations of *C. ladanifer* sampled in Morocco, Spain and France.

	Source of variation	Number of valves (analysis of deviance)			Log number of seeds (analysis of variance)			
		df	Wald χ^2	P	df	MS	F	P
Taxonomy	Subspecies	1	2.30	0.13	1	0.007	0.79	0.38
	Population (subspecies)	33	1738.43	<0.0001	18	0.240	13.68	<0.0001
	Plant (population)	458	1290.89	<0.0001	83	0.018	2.05	<0.0001
	Error				100	0.009		
Phylogeography	Haplotype	2	251.57	<0.0001				
	Population (haplotype)	23	760.70	<0.0001				
	Plant (population)	376	1097.82	<0.0001				

df = degrees of freedom; MS = mean squares. P values in bold are significant at the Bonferroni-corrected P level (0.05/3 = 0.017).

Fruit predation

Seeds were predated by at least three species of Coleoptera (Bruchidae, Scarabeidae, and Curculionidae) and one species of Lepidoptera larva (Noctuidae). Lepidoptera larva was the most predominant seed predator (83.3% populations) followed by Bruchidae (50.0% populations) and Scarabeidae and Curculionidae (33.3% populations). In one population (Val) seed predation was caused by the four insect species; in the rest, seeds were predated by two (Alg, Lar, Nor1) or one (Alh2, Boa) insect species.

Fruit infestation in *C. ladanifer* was highly variable at both the intra- and the inter-population levels. In the process of fruit infection, insects did not seem to discriminate between fruits with different numbers of valves in any population, as the number of valves of fruits affected and unaffected by seed predators was not statistically different ($P > 0.25$ for all populations).

Seed predation affected most plants in all populations; thus, the percentage of individuals per population with at least one fruit predated was higher than 85% in all populations with the exception of Alg, which had a predation rate of 50% (Table 3). Differences in fruit predation between populations with low and high numbers of fruit valves were not significant ($U = 3$, $P = 0.52$). The percentage of fruits affected per population ranged between 3.3% (Alg population) and 47.5% (Alh2, Table 3). Again, differences between populations with low and high numbers of fruit valves were not significant ($U = 2$, $P = 0.25$). In damaged fruits of all populations, the number of fruit valves affected was not statistically different among fruits with different numbers of valves (Table 4); thus, the percentage of predated valves per fruit was not related to the number of fruit valves. The mean percentage of predated valves in infested fruits per population ranged from 33.0% (Alg population) to 72.7% (Alh2, Table 3); these differences between populations were statistically significant (Wald $\chi^2_4 = 44.70$, $P < 0.0001$). Because there was high variation among populations with a similar number of fruit valves (Table 3), differences were not significant when populations with low and high numbers of fruit valves were considered (Wald $\chi^2_1 = 0.0001$, $P = 1.0$). The percentage of predated valves per fruit among plants were not statistically significant (Wald $\chi^2_{100} = 44.70$, $P = 0.27$).

Altitude and climatic factors were not correlated with the percentage of fruits predated per population (Table 5). The same was true for the percentage of affected fruit valves in damaged fruits (Table 5). Populations with haplotypes 1 and 6 showed similar percentages of predated fruits (Wald $\chi^2_1 = 1.411$, $P = 0.235$) and percentages of predated valves per fruit (Wald $\chi^2_1 = 0.022$, $P = 0.882$).

Discussion

C. ladanifer is a highly polymorphic species of Mediterranean flora that exhibits variation in leaf size and shape, flower size and colouration patterns, and ovary divisions (Demoly and Montserrat,

Table 3
Damage caused by seed predators in fruits of six populations of *C. ladanifer* with low (L) or high (H) numbers of fruit valves.

Population	Fruit valves (mean \pm SE; median)	Plants with at least one predated fruit (%)	Predated fruits (%)	Percentage of predated valves per fruit (mean \pm SE)
Alg (L)	8.3 \pm 0.26; 8	50	3.3	33.0 \pm 7.5
Alh2 (L)	7.1 \pm 0.05; 7	100	47.5	72.7 \pm 2.6
Boa (L)	8.3 \pm 0.14; 8	85	13.5	62.9 \pm 4.9
Lar (H)	9.4 \pm 0.10; 10	100	45.1	66.8 \pm 4.1
Nor1 (H)	9.9 \pm 0.04; 10	100	18.2	35.9 \pm 4.3
Val (H)	9.5 \pm 0.06; 10	95	22.3	62.3 \pm 3.0

Table 4
Results of a nested analysis of deviance comparing the percentage of predated valves per fruit among fruits with different numbers of fruit valves in six populations of *C. ladanifer*.

Population	Source of variation	Analysis of deviance		
		df	Wald χ^2	P
Alh2	Plant	19	36.60	0.009
	Valves (plant)	21	17.20	0.699
Lar	Plant	19	24.80	0.167
	Valves (plant)	26	38.16	0.059
Nor1	Plant	19	36.66	0.006
	Valves (plant)	5	16.5	0.123
Val	Plant	18	36.60	0.009
	Valves (plant)	21	17.20	0.699
Alg	Valves	2	1.56	0.461
Boa	Valves	3	1.8	0.612

In Alg and Boa populations, “valves” was not nested within “plant” because the number of infested fruits per plant was very low. See Appendix A for population codes.

1993). The number of valves per fruit (5–12) was variable at the inter-population, inter-individual, intra-individual and inter-annual levels. However, most heterogeneity was detected among populations (57.8%). To our knowledge, this is one of the first studies to detect such an extraordinary degree of variation in valves per fruit within a single species across individuals and populations over time. Our analyses indicated that no single component could explain most of the variation observed across the geographical range of this species. Instead, some analyses of fruit variation detected significant effects of ecogeographical variables and co-ancestry. Additionally, we report an important variation at the individual level, although our sample was not particularly large at this level. Intra-individual variation in reproductive traits has traditionally been ignored and high stability has been assumed, but intra-individual variation is increasingly recognised as an important component of phenotypic variability upon which selection can act (Herrera, 2009).

Table 5
Spearman rank correlations between pre-dispersal seed predation and environmental factors characteristic of the populations of *C. ladanifer*.

Variable	Variable	N	ρ	P
%Predated fruits	T	6	0.20	0.70
%Predated fruits	T _h	6	0.14	0.79
%Predated fruits	PREC	6	-0.09	0.87
%Predated fruits	Altitude	6	0.37	0.47
%Predated valves per fruit	T	6	-0.14	0.79
%Predated valves per fruit	T _h	6	-0.03	0.96
%Predated valves per fruit	PREC	6	-0.26	0.62
%Predated valves per fruit	Altitude	6	0.71	0.11

The populations used in all correlations are detailed in Appendix B. Significance levels were corrected using a sequential Bonferroni test (i.e., 0.05/4; corrected $\alpha = 0.0125$). See Appendix A for abbreviations.

Ecogeographical and phylogeographical patterns

There was a subtle, but significant, correlation between the number of fruit valves and the environmental factors. In particular, high altitude and low precipitation are correlated with a low number of fruit valves (Fig. 5). Different phenotypic optima may be expected under different environmental conditions, and the wide distribution area of *C. ladanifer* (Fig. 1A) implies exposure to a broad range of conditions. Variation in fruit parts has been studied in some Mediterranean plants, but such analyses included only a few populations of plants that displayed a lower number of carpels (2–4 in *Helleborus foetidus*: Guitián et al., 2003; 1–5 in *Paeonia broteri*: Sánchez-Lafuente, 2007) than *C. ladanifer*; thus most of the observed variance was found within populations. More importantly, a significant difference in the number of fruit valves for different haplotypes of *C. ladanifer* and a significant correlation between genetic distances and the number of fruit valves indicate that differences among populations are due, to some extent, to co-ancestry patterns or genetic relationships among populations. Although some positive correlation between phylogeography and fruit-valve variation is first reported, this result should be taken with caution due to the unbalanced number of haplotypes across the distribution range (Guzmán and Vargas, 2009), and then studied populations per haplotype (haplotypes 4 and 6 in four populations each; haplotype 1 in 17 populations; Appendix A). This indicates that fruit traits are, in part, related to the genotypes of the populations and may be independent of adaptation to current environmental conditions.

Additional factors influencing fruit and seed variation

We failed to find significant effects of taxonomic identity, geographical location, or pre-dispersal seed predation. Taxonomy was not related to variation in fruit valves for two of the three subspecies analysed. Although the taxonomic delimitation of the species is to some extent associated with haplotype distribution (Guzmán and Vargas, 2009), the phylogeographical pattern detected in the number of fruit valves is not associated to taxonomic differentiation. The most important geographical feature in the distribution of the gum rock-rose (the Strait of Gibraltar) appears not to be related to the variations in the number of valves and seeds (see Rodríguez-Sánchez et al., 2008 for a biogeographical review). The degree of pre-dispersal seed predation was highly heterogeneous among populations, as previously found in other studies (Bastida and Talavera, 2002; Metcalfe and Kunin, 2005; Delgado et al., 2007). It is, however, necessary to extend sampling across populations to test the hypothesis of increased effects of selection by insect predation for individuals bearing fruits with fewer fruit valves (Delgado et al., 2007).

The fact that our multi-level analysis only detected part of the variation due to some factors (altitude, precipitation, co-ancestry) related to fruit polymorphism leads us to consider additional factors not tested in the present study. Phenotypic plasticity may induce different responses in fruit-valve variation in individuals under different environmental conditions (Sultan, 2000). In fact,

the capacity of the same individual to modify a fruit trait (such as valve number in *C. ladanifer*) has been reported in other species, although studies at the intra-individual level are limited (Vogler et al., 1999; Hennenberg and Bruelheide, 2003; Mal and Lovett-Doust, 2005; Herrera, 2009). Furthermore, in modular organisms as *C. ladanifer* (Serrano et al., 2007), significant within individual inter-annual differences in the number of fruit valves as reported in the present study could be reflective of phenotypic plasticity in response to different environmental conditions throughout the growing season (de Kroon et al., 2005). One additional source of variation in traits of *C. ladanifer* influenced by environmental conditions is developmental instability (Alados et al., 1999); this may explain why the same plant produces fruits with different numbers of valves, as it has been already found in some other species (Ellstrand and Mitchell, 1988; Doust, 2001).

Female reproductive success

Though difficult to interpret, the remarkable variation in fruit features of *C. ladanifer* may have important consequences on maternal fitness, as discussed for other species with a variable number of carpels (Alcántara et al., 2007; Manzaneda and Rey, 2008). There was also high variation in the number of seeds per fruit in the gum rock-rose; the inter-population seed variance was even greater (73.9%) than valve variation (57.8%). We did not detect a trade-off between the number of ovules and seeds per valve versus the number of valves per fruit in the studied populations. Thus, fruits with a high number of valves produced a higher number of ovules and seeds. These results are in agreement with those obtained for a single population of *C. ladanifer* (Delgado et al., 2007) and for *H. foetidus* (Gutián et al., 2003). Thus, *C. ladanifer* populations with a high production of valves (and thus ovules) per flower may have an advantage. However, our results show that the number of fruit valves does not appear to have a clear effect on maternal fitness. Fruits with a high number of valves produced a higher number of seeds, but the total fruit production per plant was not correlated with the number of fruit valves. In turn, reproductive output of *C. ladanifer* seems to be positively correlated with plant age (Talavera et al., 1993; Alados et al., 1999). The results herein presented, coupled with previous phylogenetic and phylogeographical results (Guzmán, 2008; Guzmán and Vargas, 2009), allow us to hypothesise that we are witnessing the early stages of a differentiation process of *C. ladanifer*.

Conclusions

Our study detected a number of factors related to fruit and seed variation in *C. ladanifer*, thus introducing new lines of investigation. Further developmental, genetic and ecological studies are needed to elucidate the causes of the extraordinary variability found in the number of fruit valves in *Cistus*. In particular, studies under controlled (common garden) conditions or reciprocal transplants in contrasted natural conditions are essential for testing the effects of plasticity and developmental instability. Plasticity of vegetative traits has been extensively studied across populations and a theoretical background has been proposed to explain the adaptive potential (e.g., in highly spatially/temporally variable environments, Valladares and Niinemets, 2008). However, there is no comparable approach to explain variability in reproductive traits within a single individual (but see Vogler et al., 1999; Dorken and Barrett, 2004; Mal and Lovett-Doust, 2005; Brock and Weinig, 2007), although in principle the same rules may apply (Herrera, 2009). In summary, our study not only supports the early predictions by Saint-Hilaire and Darwin that the multiplicity of flower parts is "... very numerous, the number is generally variable" (Darwin, 1859), but also suggests a more complex evolutionary process of trait multiplicity.

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Appendix A.

Sites (ordered by decreasing latitude), geographical parameters, climatic characteristics and infra-specific level characteristics of the studied populations of *C. ladanifer*. Populations analysed in the altitude study in bold.

Code	Locality	Subspecies/haplotype*	Latitude	Longitude	Altitude (m a.s.l.)	T (°C)	T _h (°C)	PREC (mm)
Mon	Montpellier, Alsace, France	<i>ladanifer</i> /1	43°36'N	3°52'W	185	13.9	22.2	710
Lar	Laroco, Orense, Spain	<i>ladanifer</i> /1	42°21'N	7°10'W	512	10.5	18.0	927
Zam	Peñausende, Zamora, Spain	<i>ladanifer</i> /1	41°14'N	5°53'W	960	13.2	23.6	443
Sal	Valdunciel, Salamanca, Spain	<i>ladanifer</i> /1	41°09'N	5°41'W	844	11.8	21.2	459
Ata	El Atazar, Madrid, Spain	<i>ladanifer</i> /1	40°55'N	3°28'W	1000	12.5	22.7	692
Cab	La Cabrera, Madrid, Spain	<i>ladanifer</i> /1	40°52'N	3°37'W	1000	12.5	22.7	692
Gua1	La Barranca, Madrid, Spain	<i>ladanifer</i> /1	40°45'N	4°00'W	1400	6.5	16.3	1170
Man	Manzanares el Real, Madrid, Spain	<i>ladanifer</i> /1	40°43'N	3°52'W	900	12.7	23.7	725
Esc	El Escorial, Madrid, Spain	<i>ladanifer</i> /1	40°34'N	4°07'W	900	13.4	24.2	637
Can	Tres Cantos, Madrid, Spain	<i>ladanifer</i>	40°34'N	3°42'W	700	12.7	23.7	725
Gua2	El Pardo, Madrid, Spain	<i>ladanifer</i> /1	40°30'N	3°45'W	678	13.9	24.2	438
Boa	Boadilla del Monte, Madrid, Spain	<i>ladanifer</i> /1	40°23'N	3°52'W	650	12.9	24.6	404
Cha	Chapinería, Madrid, Spain	<i>ladanifer</i> /1	40°22'N	4°12'W	600	12.9	24.6	404
Jer	Jerte, Cáceres, Spain	<i>ladanifer</i> /1	40°16'N	6°06'W	868	14.4	23.8	1138
Lin	Linares, Jaén, Spain	<i>ladanifer</i>	38°08'N	3°43'W	350	17.6	28.3	541
Nav	Las Navas de la Concepción, Sevilla, Spain	<i>ladanifer</i>	37°52'N	5°24'W	350	14.5	22.9	813
Nor2	S ^o Norte, Sevilla, Spain	<i>ladanifer</i>	37°55'N	5°34'W	803	14.5	22.9	813
Nor1	S ^o Norte, Sevilla, Spain	<i>ladanifer</i> /1	37°54'N	5°25'W	322	14.5	22.9	813
Hig	Higuera de la Sierra, Huelva, Spain	<i>ladanifer</i>	37°49'N	6°27'W	600	14.5	24.6	1026
Peñ	Peñaflor, Sevilla, Spain	<i>ladanifer</i>	37°45'N	5°22'W	270	17.5	27.6	687
Val	Valverde del Camino, Huelva, Spain	<i>ladanifer</i>	37°34'N	6°45'W	250	19.2	28.3	782
Hin	Hinojos, Huelva, Spain	<i>ladanifer</i> /1	37°17'N	6°22'W	80	17.1	26.5	618
Alh2	S ^o Alhamilla, Almería, Spain	<i>ladanifer</i> /6	36°59'N	2°22'W	1294	17.9	26.9	221
Isl	Isleta del Moro, Almería, Spain	<i>ladanifer</i> /6	36°52'N	2°00'W	370	17.3	25.9	268

Appendix A (Continued)

Code	Locality	Subspecies/haplotype*	Latitude	Longitude	Altitude (m a.s.l.)	T (°C)	T _h (°C)	PREC (mm)
Alh1	Poyatos, Almería, Spain	<i>ladanifer</i>	36°45'N	2°09'W	240	17.3	25.9	268
Alg	Algar, Cádiz, Spain	<i>ladanifer</i> /6	36°38'N	5°39'W	480	17.7	25.9	728
Ber2	S ^a Bermeja, Málaga, Spain	<i>africanus</i> /6	36°34'N	5°15'W	568	14.7	23.3	1189
Ber1	S ^a Bermeja, Málaga, Spain	<i>africanus</i>	36°32'N	5°10'W	944	14.7	23.3	1189
Asi	Asilah, Tánger, Morocco	<i>africanus</i> /4	35°46'N	5°55'W	20	–	–	–
Chao	Chaouen, Chaouen, Morocco	<i>africanus</i>	35°04'N	5°14'W	570	17.4	31.9	944
Bab	Bab-Taza, Chaouen, Morocco	<i>ladanifer</i> /1	35°04'N	5°10'W	1050	14.9	32.3	1482
Dje	Djebel Bouhalia, Chaouen, Morocco	<i>ladanifer</i> /1	35°04'N	5°10'W	600	14.9	32.3	1482
Ben	Beni-Hadifa, Al Hoceima, Morocco	<i>africanus</i> /4	35°01'N	4°09'W	1065	15.2	30.6	900
Tar	Targhist, Al Hoceima, Morocco	<i>africanus</i> /4	34°57'N	4°21'W	1240	14.4	30.8	300
Ket	Ketama, Al Hoceima, Morocco	<i>africanus</i> /4	34°53'N	4°35'W	1400	11.4	26.9	900
Taf	Taforalt, Oudja, Morocco	<i>africanus</i> /7	34°49'N	2°29'W	874	–	–	–

T, mean annual temperature; T_h, mean temperature of the hottest month; PREC, mean annual precipitation. Climatic data from the Worldwide Bioclimatic Classification System (<http://www.ucm.es/info/cif/data/indexc.htm>).

* GenBank accession number in Guzmán and Vargas (2009) except GenBank accession number for Alg (HM773247).

Appendix B.

Material used for the multi-level variation analysis of *Cistus ladanifer*. Number of studied populations and individuals from SW Europe and northern Africa. Populations codes as in Appendix A.

Study level	Number of populations	Number of individuals per population	Population codes
Valve number			
Overall variation	34	6–21	All populations except Man, Nor2
Temporal (two years)	18	6–20	Lar, Sal, Ata, Cab, Esc, Can, Gua1, Gua2, Boa, Cha, Jer, Nor1, Hig, Val, Alh2, Isl, Alh1, Ber
Temporal (three years)	6	20–40	Can, Gua, Boa, Cha, Alh1, Alh2
Geographical	34	6–21	All populations except Man, Nor2
Ecogeographical	34	6–21	All populations except Man, Nor2
Taxonomic	34	6–21	All populations except Man, Nor1
Phylogeographical	25	7–20	Mon, Lar, Zam, Sal, Ata, Alg, Cab, Man, Esc, Gua1, Gua2, Boa, Cha, Jer, Nor1, Hin, Alh2, Isl, Ber2, Asi, Bab, Dje, Ben, Tar, Ket
Seed number			
Overall variation	19	4–16	Lar, Zam, Ata, Cab, Man, Esc, Boa, Cha, Lin, Nav, Nor1, Peñ, Val, Hin, Alh2, Ber, Chao, Bab, Ket
Geographical	19	4–16	Idem
Ecogeographical	19	4–16	Idem
Taxonomic	19	4–16	Idem
Trade-off estimate			
Ovules/valves	4	20	Cab, Esc, Nor1, Ber1
Seeds/valves	4	20	Cab, Esc, Nor1, Ber1
Total fruits/valves	4	9–32	Nor1, Nor2, Ber1, Hin
Fruit and seed predation			
Intra- and inter-population	6	20	Alg, Alh2, Boa, Lar, Nor1, Val

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