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CENSUSING NATURAL MICROGAMETOPHYTE POPULATIONS: VARIABLE SPATIAL MOSAICS AND EXTREME FINE-GRAININESS IN WINTER-FLOWERING *HELLEBORUS FOETIDUS* (RANUNCULACEAE)[1]

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Little is known about patterns and correlates of variation in microgametophyte populations for naturally pollinated plants, yet this information is critical for evaluating the prevalence and potential evolutionary significance of gametophyte competition in the wild. This paper analyzes spatial and temporal variation in microgametophyte populations (= number of pollen tubes per style) for the winter-flowering, perennial herb *Helleborus foetidus* (Ranunculaceae), based on data from 29 populations in three regions of the Iberian Peninsula collected over two consecutive years. Mean size of microgametophyte populations varied significantly at a wide range of spatial scales, including among regions, among populations within regions, among individual plants within populations, among flowers of the same plant, and among pistils of the same flower (*H. foetidus* flowers are apocarpous). Differences between regions were quantitatively negligible. Differences between populations in the same region were moderate to low, and their sign and magnitude were inconsistent between years. Roughly half of total variance in microgametophyte numbers was accounted for by variation within individual plants, and the largest part of this component was due to differences between the pistils of the same flower. These results reveal extreme spatial fine-graininess and marked stochasticity in the spatial variation of *H. foetidus* microgametophyte populations and suggest that opportunities for consistent selection on male gametophyte competitive ability are probably negligible in this species.

Key words: gametophyte competition; geographical variation; *Helleborus foetidus*; microgametophyte populations; pollen limitation; pollen tube numbers; Ranunculaceae; winter flowering.

The amount of conspecific pollen deposited on the stigma of angiosperm flowers influences both the number and the quality of the progeny. In addition to setting an obvious upper limit to the maximum number of ovules that can be fertilized, the number of pollen grains on the stigma may also influence the number of seeds produced through a complex combination of factors involving threshold effects, species composition, nonlinear dose-response relationships, and maternal and paternal identity (e.g., Snow, 1982; Schemske and Fenster, 1983; Waser and Fugate, 1986; Bertin, 1990; Waser and Price, 1991; Holm, 1994; Plitmann and Levin, 1996; Mitchell, 1997a; Bosch and Waser, 1999). The amount of pollen may also influence the quality of the progeny (Mitchell, 1997b; Winsor, Peretz, and Stephenson, 2000; and references therein), an effect that may be mediated by both pre-fertilization (competition among male gametophytes; Lee, 1984; Snow, 1986; Schlichting et al., 1987; Winsor, Davis, and Stephenson, 1987; Winsor, Peretz, and Stephenson, 2000) and post-fertilization mechanisms (selective abortion exerted by the pistil-bearing individual; Willson and Burley, 1983; Stephenson and Winsor, 1986; Marshall and Ellstrand, 1988; Niesenbaum and Casper, 1994; Rigney, 1995; Havens and Delph, 1996; Niesenbaum, 1999).

From a biological viewpoint, however, pollen load size is only a convenient proxy for the parameter most directly influencing seed production and progeny quality, namely the number of pollen tubes that penetrate the stigma and enter the transmitting tissue of the style (designated ''microgametophytes'' hereafter). It is the size of this microgametophyte population that will most directly set an upper limit to the number of fertilizable ovules and also, under some circumstances (synchrony in pollen arrival and germination; Snow, 1986; Thomson, 1989), will determine the possibilities of gametophyte competition. Pollen grain and pollen tube numbers will generally be correlated. This justifies, for example, inferences on gametophytic competition in natural populations based on observations of the number of deposited pollen grains alone (e.g., Spira et al., 1992). Nevertheless, the relationship between the number of conspecific, compatible pollen grains on the stigma and the number of pollen tubes in the style is generally a very loose one (Snow, 1986; Herrera, 1997) due, among other factors, to environmental conditions, pollen–pistil interactions, pollen tube attrition effects, and to the marked stochasticity and strong context dependence of the germinating behavior of pollen grains (Cruzan, 1986, 1989; Thomson, 1989; Stephenson et al., 1992; Holm, 1994). Therefore, although both seed set and levels of gametophyte competition levels will ultimately be related to pollen load size, the number of pollen tubes that penetrate the stigma is a more proximate variable in the reproductive success of flowers (Plitmann and Levin, 1996; Quesada, Fuchs, and Lobo, 2001).

In comparison with the multitude of manipulative experiments examining the effects of variations in the size of stigmatic pollen loads, only a handful of investigations have so far provided information on patterns and correlates of variation in microgametophyte populations for naturally pollinated plants in the wild (Levin, 1990; Honig, Linder, and Bond,

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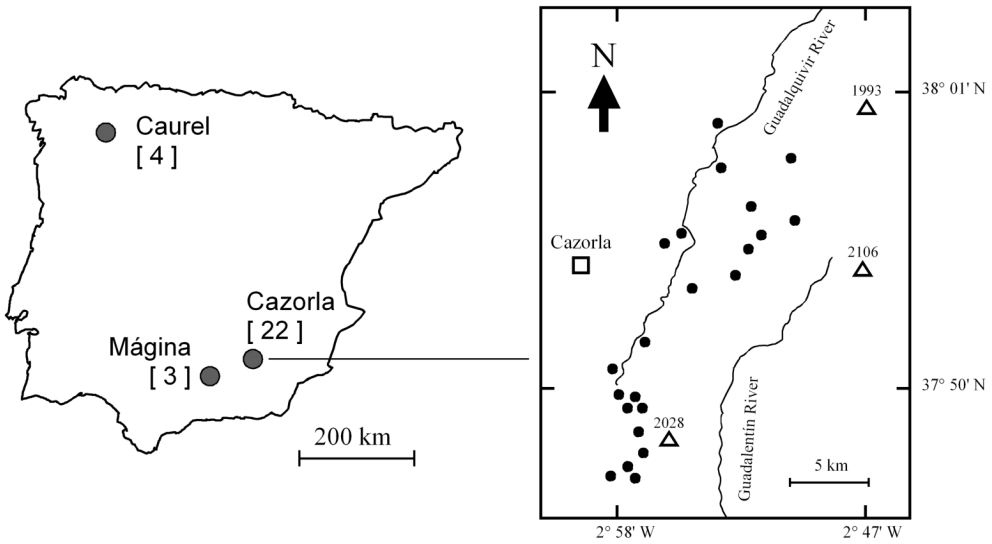

Fig. 1. Map of the Iberian Peninsula (left) showing the location of the three regions considered in this study. Figures in brackets indicate the number of populations sampled in each region. The map on the right shows the distribution of the populations studied in the Cazorla region (filled dots).

1992; Niesenbaum, 1994; Plitmann and Levin, 1996; Herrera, 1997; Quesada, Fuchs, and Lobo, 2001). Direct measurements of the size of natural microgametophyte populations and its relationship to ovule number are essential to assess where individual plants, populations, or species stand along the conceptual gradient running from extreme pollination deficit (microgametophyte/ovules ratio $\ll 1$) to potentially intense competition between gametophytes (microgametophyte/ovules ratio $\gg 1$). Furthermore, knowledge of natural patterns of spatial and temporal variation in microgametophyte numbers is a prerequisite for evaluating the prevalence and biological significance in naturally pollinated plant populations of the mechanisms and effects elucidated by experimental manipulations of pollen loads, including gametophyte competition. Documenting temporal or geographical variation in microgametophyte populations for a given species, for instance, may provide insights on possible spatiotemporal mosaics in the degree of pollen limitation and intensity (or likelihood) of gametophyte competition and associated selective processes. Furthermore, information on the hierarchical apportionment of variance in per-pistil microgametophyte populations among and within individual plants is essential to evaluate the actual opportunities of selection on any influential plant trait. Of particular relevance from an evolutionary perspective are the relative magnitudes of within- vs. between-individual variance in number of microgametophytes per pistil, as the possibilities of natural selection to act on whichever trait is influential on pollen tube numbers would be directly related to the fraction of total variance accounted for by between-individual differences. In one of the few studies examining patterns of variation of microgametophyte populations among individual plants in the wild, Niesenbaum (1994) found that, in *Lindera benzoin*, as little as 7% of total population-wide variance in pollen tube numbers was accounted for by differences among individual plants. This intriguing finding raises doubts on the opportunities for evolutionary change of any trait correlated with pollen tube numbers in that species and calls for confirmation in other plants.

The present study was undertaken to assess patterns of spatial and temporal variation in microgametophyte numbers in the style of naturally pollinated flowers of the winter-flowering, perennial herb *Helleborus foetidus* (Ranunculaceae). The main goals of this paper are (1) to provide a comprehensive picture of the magnitude and scales of spatial and temporal variation in microgametophyte populations for this species and identify the main sources of variation in microgametophyte numbers and (2) to evaluate the relative frequency of occurrence of situations of pollen deficit and gametophyte competition, information that is still hardly available for any other plant species under natural conditions. A series of hierarchically nested spatial scales spanning several orders of magnitude is considered, ranging from hundreds of kilometers (between separate regions within the Iberian Peninsula) through a few kilometers (between populations in the same region) to individuals within populations (dozens to hundred meters), flowers within plants (centimeters) and, finally, distinct styles within individual flowers (millimeters; *H. foetidus* flowers are apocarpous, each carpel bearing a distinct style). To investigate the temporal component of variation, all the populations in the most thoroughly studied region were sampled during two consecutive years.

MATERIALS AND METHODS

Study system and sites—*Helleborus foetidus* L. (Ranunculaceae) is a perennial herb widely distributed in western Europe (Werner and Ebel, 1994). In the Iberian Peninsula, the species typically grows in the understory of deciduous and mixed forests. Flowers are hermaphroditic, protogynous, last for 2–3 wk, and the stigmas are receptive for 6–12 d (Vesprini, Nepi, and Pacini, 1999; Vesprini and Pacini, 2000; Herrera et al., 2001). Flowers are apocarpous, with the number of carpels ranging between one and six (mostly 2–3) per flower. Pollination is performed by bumble bees and, to a lesser extent, anthophorid bees (Herrera et al., 2001). Although the species is self-compatible and a small proportion of flowers set fruit in the absence of pollinators via spontaneous self-pollination, insect pollination is required for abundant seed production (Vesprini and Pacini, 2000; Herrera et al., 2001).

This study was conducted in 2000 and 2001 at three widely separated regions in the Iberian Peninsula (Fig. 1). The two most distant regions (Caurel and Cazorla) were ~675 km apart, while the two nearest ones (Mágina and Cazorla) were only ~85 km away. The flowering period was roughly similar at the three study regions, extending mainly from January to March (in some

TABLE 1. Summary of linear regression analyses relating the total number of seeds produced per individual follicle (''Follicle level'' analysis) or fruit (all follicles from the same flower combined; ''Fruit level'' analysis) and the number of pollen tubes in the corresponding styles at flowering time, for three *Helleborus foetidus* populations in Cazorla. All regressions are statistically significant at $P < 0.0001$. Regressions were forced through the origin because of the structural constraint that zero pollen tubes should produce zero seeds.

Population no.	Elevation (m)	Follicle level[a]			Fruit level[a]		
		N	*b* ± SE	R^2	*N*	*b* ± SE	R^2
11	760	172	0.737 ± 0.05	0.60	79	0.731 ± 0.07	0.61
19	1675	159	0.738 ± 0.04	0.65	66	0.735 ± 0.06	0.67
15	1800	218	0.843 ± 0.04	0.71	90	0.857 ± 0.06	0.72

[a] *N* = sample size, *b* = linear regression coefficient, SE = standard error, R^2 = adjusted coefficient of determination.

Cazorla localities at low elevations, flowering started in December). A variable number of *H. foetidus* populations were selected for study in each region: four populations in Caurel (elevation range = 950–1350 m), three populations in Mágina (elevation range = 1640–1670 m), and 22 populations (elevation range = 760–1800 m) in Cazorla. At the northwestern region (Caurel), the *H. foetidus* populations chosen for study grew in pine (*Pinus sylvestris*) plantations, open successional scrublands, and *Brachypodium rupestre* meadows. At the two southern regions (Cazorla and Mágina) the selected populations were in pine- (*Pinus nigra*) or oak- (*Quercus rotundifolia*) dominated forests. Cazorla populations encompassed and were more or less evenly distributed over the whole elevational range of *H. foetidus* in the region. Caurel and Mágina populations were studied only in 2000. The 22 Cazorla populations were studied in 2000 and 2001, and the extensive data from this region provided the core information for this study. Information on the pollination ecology of *H. foetidus* at the three study regions is given by Herrera et al. (2001).

Field and laboratory methods—During March–April, styles were sampled from open or newly withered flowers at all study populations. To account for elevational differences in flowering phenology (particularly in Cazorla), the sampling date at each population was adjusted so as to roughly coincide with the local flowering peak. Between 6 and 12 styles were collected from each of ten different plants in each population and stored in microcentrifugue tubes filled with 2.5–2.5–95% formaldehyde-acetic acid-ethyl alcohol solution (FAA). To ensure that pollen tube counts reflected the final number of pollen tubes in the style at the end of the female period of each flower, only styles from either newly withered flowers or open flowers that had already completed their female stage (showing nonreceptive, dark stigmas) were collected. In most populations and sampling occasions, all styles collected from the same plant were pooled into per-plant batches. The within-flower component of variation in pollen tube numbers (i.e., among styles within the same flower) was assessed in 2000 in the four Caurel populations and in three populations in Cazorla. In these instances, styles from different flowers of the same plant were kept in separate vials.

Martin's (1959) epifluorescence method was used to reveal pollen tubes in the collected styles. Styles were kept at 65°C for 20 min in 5 mol/L NaOH for softening, rinsed in distilled water, and stained for 20 min at 65°C in decolorized aniline blue. The number of pollen tubes that had penetrated the stigma and were in the stylar canal was then counted under a fluorescence microscope. Tubes were counted on or near the midpoint between the stigma and the bifurcation of the transmitting tissue that leads to the two marginal placentae (see Weberling [1989, pp. 139–140] for drawings and descriptions of gynoecium structure in *H. foetidus*).

Assessing the implications of variation in the number of pollen tubes requires information on the size of the ovule complement of each carpel and on the effect of pollen tube numbers on seed and fruit set. Detailed information on the number of ovules per carpel was available for several populations in each of the three regions, obtained in the course of other investigations on *H. foetidus*. The range of variation of the number of ovules per carpel was very narrow both among regions (mean ± 1 SD = 11.1 ± 1.2 [*N* = 1313 carpels], 11.3 ± 1.1 [*N* = 438], and 10.8 ± 1.4 [*N* = 1025] ovules/carpel in Caurel, Cazorla, and Mágina populations, respectively) and within regions (interquartile range = 10–12 ovules/carpel at each of the three regions). An average figure of 11 ovules per carpel will thus be used throughout this paper. The relationship between number of pollen tubes and seed set was investigated in three Cazorla populations. In May 2000, samples of developing fruits (= the set of follicles originating from the same gynoecium) were collected in three populations. At each site, between six and ten fruits were collected from each of ten plants and preserved in FAA. For every follicle, the number of pollen tubes in the persistent, dry style was counted using the same epifluorescence technique described above, along with the number of enclosed undeveloped ovules and nearly ripe seeds.

TABLE 2. Summary statistics for the number of pollen tubes in styles of *Helleborus foetidus* at the three study regions (see Fig. 1 for location), based on combined data from four (Caurel), three (Mágina), and 22 (Cazorla) populations. *N* = number of styles examined.

Region	Year	*N*	Interquartile range	Mean ± 1 SD	Percentage styles with <11 tubes[a]
Caurel	2000	288	8–11	9.4 ± 2.3	67.0
Mágina	2000	210	7–10	8.3 ± 2.8	81.0
Cazorla	2000	1269	8–13	10.8 ± 3.7	52.0
	2001	1536	7–15	10.8 ± 6.3	39.6

[a] Flowers of *H. foetidus* have an average of 11 ovules per carpel.

Statistical analyses—All statistical analyses were carried out using the SAS statistical package. Variance components of microgametophyte numbers at the various spatial scales considered were estimated using the restricted maximum likelihood method, as implemented in the SAS procedure MIXED (SAS, 1996). This procedure also provides approximate standard errors of variance component estimates and two-tailed tests of significance based on standard normal deviates.

RESULTS

Pollen tubes and seed set—In the three Cazorla populations where number of pollen tubes in the styles and seed production were determined concurrently, there was a highly significant, positive linear relationship between the two variables, irrespective of whether the analysis was conducted at the per-follicle or per-fruit level (Table 1). At the three populations, variation between carpels and between flowers in pollen tube numbers explained a substantial fraction of variance (60–70%) in the number of seeds produced eventually. The magnitude of the regression coefficient relating seed production to pollen tube number differed very little among populations or computation methods. On average, every additional pollen tube in the style was associated with an increase of ~0.73–0.86 in the number of seeds produced (Table 1).

Variation between regions—In 2000, there was significant heterogeneity among regions (Caurel, Mágina, and Cazorla) in mean number of pollen tubes per style ($\chi^2 = 110.9$, df = 2, $P < 0.0001$; Kruskal-Wallis analysis of variance, data from all populations in each region pooled). In absolute terms, however, the magnitude of differences between regional means was quite small (Table 2), with the largest and smallest means (Cazorla and Mágina, respectively) differing by only two pollen tubes. Within-regional variability, as assessed by the interquartile ranges and the proportion of styles with a number of

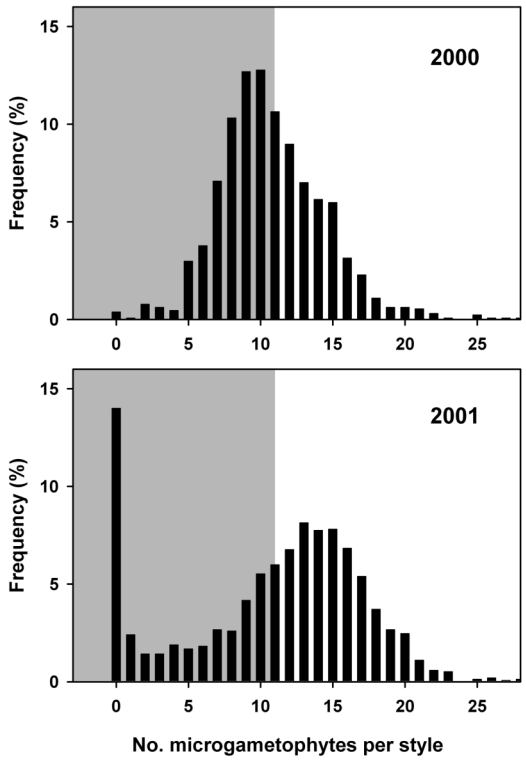

Fig. 2. Frequency distributions of the number of microgametophytes per style in the Cazorla region in 2000 and 2001, all populations combined. See Table 2 for sample sizes and summary statistics. In each graph, the shaded portion denotes the area of the distribution with number of pollen tubes inferior to the mean number of ovules per carpel (11).

pollen tubes smaller than the number of ovules, were also roughly similar in the three regions in 2000.

Regional and annual variation—Frequency distributions of microgametophyte numbers in the Cazorla region, the 22 populations combined, are shown in Fig. 2. Although the summary statistics for this region were virtually identical for 2000 and 2001 (Table 2), the shapes of the frequency distributions differed markedly between years ($D = 0.192$, $P < 0.0001$; Kolmogorov-Smirnov two-sample test). In 2000 the distribution was approximately normal and centered around the mean, while it was clearly bimodal in 2001, which reveals contrasting situations in the two study years. The proportion of styles without any pollen tube increased from 0.4% in 2000 to 14.0% in 2001, yet the proportion of styles with number of pollen tubes ≥ 11 (= the mean number of ovules per carpel) increased from 48.0% in 2000 to 60.4% in 2001.

In each study year, Cazorla populations were significantly heterogeneous with regard to the mean number of pollen tubes per style ($\chi^2 = 180.4$, df = 21, $P < 0.0001$, for 2000; $\chi^2 = 527.0$, df = 21, $P < 0.0001$ for 2001; Kruskal-Wallis analysis of variance, data from all individuals in each population pooled). Population means ranged between 6.9 and 13.2 tubes in 2000, and between 1.9 and 15.5 tubes in 2001 (Fig. 3), and there was a statistically significant increase in the variability of population means from 2000 to 2001 ($P < 0.0001$, Levene's test).

The sign and magnitude of differences among population means exhibited little consistency between years, as revealed by multiple discordances in the ranking of populations (Fig. 3) and by the weak and only marginally significant correlation of population means between years ($r_s = 0.404$, $N = 22$, $P = 0.06$). This was largely attributable to the fact that the shape of the relationship between population means and elevation differed markedly between years. While population means and elevation were not significantly related in 2000 ($r = 0.049$, $N = 22$, $P = 0.83$), there was a significant decline of mean microgametophyte numbers with increasing elevation in 2001 ($r = -0.608$, $N = 22$, $P = 0.002$) (Fig. 4). This change in the shape of the elevational gradient was mainly caused by a generalized decrease of microgametophyte means at highland populations from 2000 to 2001 (Fig. 4). In some of the populations located above 1500 m, more than half of the styles did not contain any pollen tubes in 2001 (data not shown), which accounts for the bimodal frequency distribution for all populations pooled in 2001 (Fig. 2).

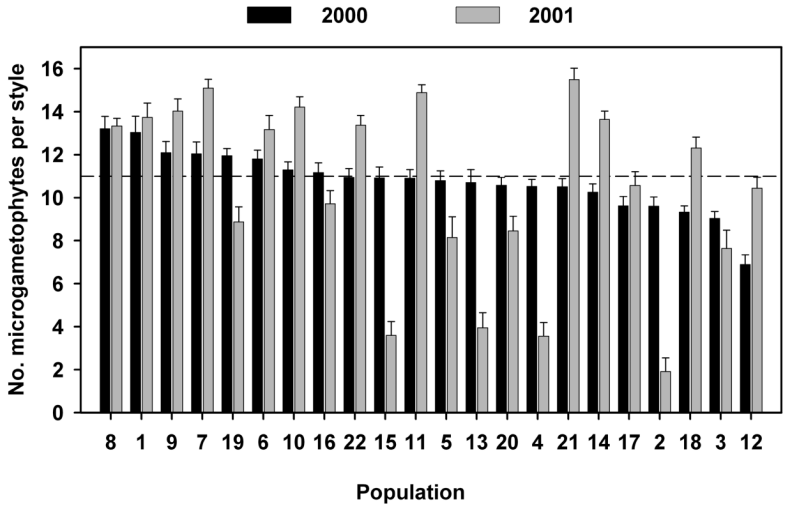

Fig. 3. Variation among Cazorla populations in mean number of microgametophytes per style in 2000 (filled bars) and 2001 (shaded bars). From left to right, populations are arranged in decreasing order of mean values for 2000. Vertical segments extend over 1 SE. The horizontal dashed line is shown as a reference for the mean number of ovules per carpel ($y = 11$).

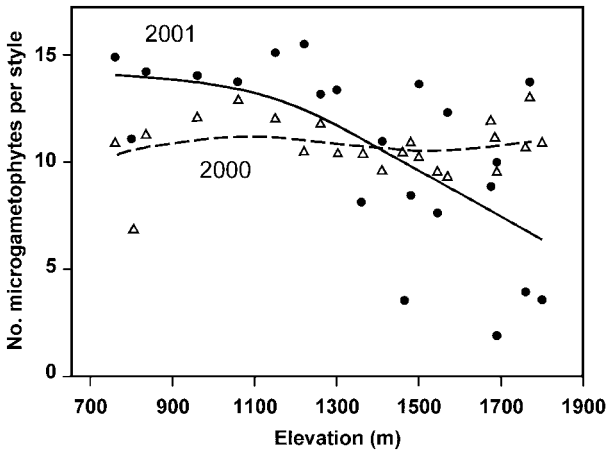

Fig. 4. Variation in mean number of microgametophytes per style over the elevational range of *Helleborus foetidus* in the Cazorla region in 2000 (triangles and dashed line) and 2001 (circles and solid line). Symbols correspond to population means in a given year, and lines are smoothing splines fitted to the data.

Variation among plants—Significant variation among individual *H. foetidus* plants in mean size of microgametophyte populations was the rule in the populations studied. Differences among plants in mean pollen tube numbers were statistically significant in the three Mágina populations, in three out of the four Caurel populations, and in 20 out of the 22 Cazorla populations in each 2000 and 2001 ($P < 0.05$ or better; tested with separate Kruskal-Wallis ANOVAs for each population and year, results not shown). In the two years and at all populations, there were individual plants with mean microgametophyte numbers above and below 11, the mean number of ovules per carpel. In most Cazorla populations, the magnitude of differences in mean tube numbers between extreme individuals was in the order of 5–8 tubes, but there was a trend towards greater within-population variability in 2001 (Fig. 5). This was tested by computing for each population and year the coefficient of variation of plant means, and then performing a paired comparison of population variability values for 2000 and 2001. Individual variability within populations in mean pollen tube numbers increased significantly from 2000 (mean CV = 22.4%) to 2001 (mean CV = 40.7%) ($t = 2.60$, $N = 22$, $P = 0.016$; paired Student's t test).

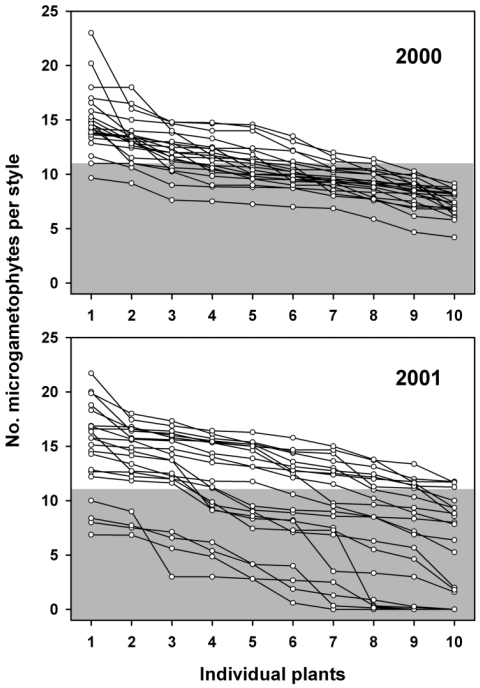

Fig. 5. Within-population, among-individual variation in mean number of pollen tubes per style in the 22 Cazorla populations studied in 2000 and 2001. Each symbol corresponds to the mean for an individual plant, and means from the same population are connected by a line. In each population, plants have been ranked on the horizontal axis in decreasing order of mean number of pollen tubes. The shaded portion of each graph denotes the area with number of pollen tubes less than the mean number of ovules per carpel (11).

Partitioning variance in microgametophyte numbers—The 2000 data for the three regions allowed for partitioning the total variance in microgametophyte numbers into components due to variation among regions, among populations within region, among plants within population, and within plants, this latter component incorporating the variance due to variation among flowers and among styles within the same flower (Table 3). Variation among regions and among populations within regions accounted for only a minimal proportion of total variance in microgametophyte numbers (10.1% and 8.5%, respectively). Most variance was accounted for by variation among plants within population (30.9%) and, principally,

TABLE 3. Partition of variance in the number of pollen tubes per style in some of the data sets examined in this study. Entries in the table represent the variance component accounted for by a given source of variation (row) in a given data set (column), with the standard error shown in parentheses. Dashes denote variance components that either are not applicable or could not be computed for that particular data set. Variance components were tested for significant differences from zero using an approximate asymptotic test (for details, see Materials and Methods: Statistical analyses; * $P < 0.05$ or better; ns not significant).

Source	All regions 2000	Data subsets with within-flower sampling		Cazorla[c]	
		Cazorla 2000[a]	Caurel 2000[b]	2000	2001
Region	1.35 (1.64)ns	—	—	—	—
Population	1.13 (0.47)*	0.03 (0.18)ns	0.52 (0.57)ns	1.24 (0.59)*	16.67 (5.46)*
Plant	4.12 (0.48)*	0.76 (0.42)*	0.75 (0.45)*	5.31 (0.66)*	6.80 (0.98)*
Within-plant (total)	6.71 (0.25)*	9.10 (0.55)*	4.51 (0.36)*	7.07 (0.31)*	18.71 (0.73)*
Flower	—	3.60 (0.63)*	2.02 (0.51)*	—	—
Carpel	—	5.50 (0.44)*	2.49 (0.28)*	—	—

[a] Three populations.
[b] Four populations.
[c] Twenty-two populations.

within plants (50.4%). The variance component due to variation among regions was not significantly different from zero, thus further stressing the negligible importance of this source of variance.

Within-plant variance in microgametophyte numbers could be further dissected into its among-flower and within-flower (i.e., among carpels) components for the three Cazorla and four Caurel populations with sufficiently detailed data (Table 3). In these data sets, within-flower variance accounted for as much as 55.6% (Cazorla) and 43.1% (Caurel) of total regional variance, variance among flowers ranked second in importance (36.4 and 34.9% in Cazorla and Caurel, respectively), and components due to variation among plants and among populations were negligible (in the case of the among-population component, not significantly different from zero).

In the 22 Cazorla populations, the variance partition profile differed between years in significant ways. The component due to variation among populations increased considerably from 2000 to 2001 in both absolute (1.2–16.7) and relative (from 13.6–39.5%) terms, which reflects the marked annual differences in the degree of variability of population means noted earlier (Fig. 3). The within-plant variance component more than doubled from 2000 to 2001 in absolute terms (7.1–18.7) and remained roughly constant in relative terms (51.9–44.4%).

DISCUSSION

Winter flowering and pollination deficit—In the mountain habitats studied, harsh weather conditions prevail during most of the flowering period of *H. foetidus*. Temperatures are uniformly low, and snowfalls and extended rainy periods are frequent. Under these circumstances, insect pollinator activity is seriously limited and visitation rates to *H. foetidus* plants are extremely low (Herrera et al., 2001). Despite this, however, fruit set of naturally pollinated plants reaches relatively high levels (60–80%), quite similar to the average value reported by Sutherland and Delph (1984) for a large sample of hermaphroditic self-compatible species (72.5%).

The high fruit set of *H. foetidus* has been interpreted in relation to the long duration of their flowers, since even with flower visitation probabilities as low as those observed, the probability of individual flowers receiving at least one effective pollinator visit during its 6–15 d long female receptive stage is far from negligible (Herrera et al., 2001). Supplemental hand pollinations of open-pollinated flowers conducted in 1998 and 1999 consistently resulted in modest increases of fruit set at the three study regions, although results reached statistical significance only in Cazorla (Herrera et al., 2001: Table 2). Two results of the present study corroborate these earlier findings and suggest that *H. foetidus* experiences a weak, chronic pollination deficit at the three study regions. First, the number of pollen tubes was inferior to the number of ovules per carpel in a large proportion of styles. And second, the number of seeds produced was closely and lineary related to the number of microgametophytes. Annual fluctuation in the frequency of styles with <11 pollen tubes in the Cazorla region (52 and 40% in 2000 and 2001, respectively) suggests, however, that the magnitude of the pollination deficit may vary between years, most likely in connection with variation in weather conditions, as discussed below.

A similar combination of high natural fruit set, weak but consistent pollen limitation, and slight but significant annual fluctuation in the magnitude of the limitation, has been also found in *Narcisus longispathus* (Amaryllidaceae), another early-blooming, insect-pollinated perennial herb from the Cazorla region characterized also by the extended longevity of its flowers (Herrera, 1995). Combinations of relatively high fruit set levels, moderate to weak or no pollen limitation, and long floral durations have been likewise reported for other winter- and early-spring flowering herbs of disparate taxonomic affiliation (Schemske, 1977, 1978; Schemske et al., 1978; Motten et al., 1981; Motten, 1986; Murphy and Vasseur, 1995; Vesprini, Nepi, and Pacini, 1999; Ishii and Sakai, 2000; Vesprini and Pacini, 2000). Contrary to intuition, therefore, winter and early spring flowering possibly is not an ''inferior choice'' for insect-pollinated plants of the forest understory insofar as floral durations are long enough to cope with infrequent and irregular pollinator visitation.

Spatiotemporal mosaic of microgametophyte populations—Pollinator censuses conducted in 1998 and 1999 in Caurel, Mágina, and Cazorla revealed extensive regional similarity in pollinator composition, the same species of bumble bees (*Bombus terrestris* and *B. pratorum*) being the main pollinators at all sites (Herrera et al., 2001). Regions did differ significantly, however, in pollinator service, as measured by the probability per time unit of plants and individual flowers receiving a pollinator visit. The relatively minor differences between the three regions in mean microgametophyte numbers found in this study are consistent with these earlier results, as both pollinator service and mean number of microgametophytes per style were distinctly lower in Mágina than in either Caurel or Cazorla.

Elevational differences in mean size of microgametophyte populations in Cazorla may be interpreted in relation to differential pollinator activity caused by abiotic environmental factors. Bumble bees, the main pollinators of *H. foetidus*, are endotherms that can forage at relatively low ambient temperatures, but they still require a minimum threshold temperature for foraging (Heinrich, 1979), and their activity is quite susceptible to inclement weather, particularly rainy periods (Teräs, 1976; Lundberg, 1980). The range of elevations sampled in Cazorla was sufficiently broad for average weather conditions to deteriorate significantly with increasing elevation, which would lead to a reduction in bumble bee activity and a decline in microgametophyte numbers. This prediction was confirmed for 2001 but not for 2000, a discrepancy that may also be explained in terms of weather variations. The year 2001 was characterized by the decade's most rainy (at low and middle elevations) and snowy (at high elevations) January–February period, whereas the year 2000, in contrast, was characterized by the driest January–February period in the decade. With clear skies and sunny weather prevailing almost uninterruptedly during the whole flowering period of *H. foetidus* in 2000 at all populations, the absence of any elevational gradient in microgametophyte numbers that year was not surprising.

In all data sets analyzed, most variance in microgametophyte numbers occurred within local populations. Within-population variance, in turn, was apportioned quite unequally among and within individual plants. Although individual plants of the same population did differ significantly in mean microgametophyte numbers, the magnitude of these differences was relatively minor in comparison to the extent of variation occurring within plants, which was the most important source of variance. In those Cazorla and Caurel populations where within-plant variance could be partitioned into its

among- and within-flower (i.e., among styles) components, variation among styles of the same flower turned out to be the most important source of within-plant variation. These results indicate that there was considerably more variability in microgametophyte numbers over the few centimeters separating the flowers of the same *H. foetidus* plant and the few millimeters separating the styles of the same flower than among the means of populations and regions dozens or hundreds of kilometers away.

There are few comparable investigations providing data on microgametophyte variation in populations of naturally pollinated plants, but these suggest that patterns similar or even more extreme than those found in this study may be widespread. Within-plant variation was also the predominant source of variation in microgametophyte numbers in *Lindera benzoin* (85.4% of total regional variance; Niesenbaum, 1994) and *Lavandula latifolia* (78.0% of total regional variance; 15 populations sampled in the Cazorla region; C. M. Herrera, personal observations). The studies of Levin (1990) on *Phlox drummondii* and Honig, Linder, and Bond (1992) on the wind-pollinated *Staberoha banksii* showed as much or more variation among pistils of single plants as among plants, although no formal variance partitioning analyses were conducted in these investigations. Taken together, results from these investigations and those reported here for *H. foetidus* suggest that, in naturally pollinated populations, variation in microgametophyte numbers is a phenomenon that predominantly takes place at the within-plant scale and is thus presumably due to stochastic variations among flowers and styles in the size and composition of pollen loads. On the practical side, marked fine-graininess in the spatial structure of variance in microgametophyte populations implies that an adequate understanding of patterns of natural variation requires carefully designed sampling schemes that allow for precise partitioning of variability at the scale of individual plants and below. This finding also has implications in relation to the frequency of occurrence and ecological and evolutionary significance of gametophyte competition in natural populations, as discussed in the next section.

Fine-grained mosaics and microgametophyte competition—For all populations and years combined, styles of *H. foetidus* contained an average of 0.95 microgametophytes per ovule (M/O ratio hereafter). This figure is inferior to all the M/O values reported by the few comparable investigations on naturally pollinated plants: 1.5 in *Lindera benzoin* (Niesenbaum, 1994), ~2 in *Epilobium canum* (Snow, 1986), ~3 in *Polemonium viscosum* (Galen and Newport, 1988), 4.7 and 3.7 in *Phlox drummondii* (Levin, 1990; Plitmann and Levin, 1996), and 5.2 in *Staberoha banksii* (Honig, Linder, and Bond, 1992). Pooled average values of this kind have been used sometimes as rough indications of the possibilities of gametophyte competition in natural populations, and the M/O ratio <1 found here for *H. foetidus* would tend to rule out the possibility of gametophyte competition in this species. Nevertheless, extensive spatiotemporal variability in microgametophyte numbers revealed by this and other studies (Levin, 1990; Plitmann and Levin, 1996) suggest that average M/O figures may convey little information on the possible occurrence of gametophyte competition and should be interpreted with caution. At least in *H. foetidus*, there is evidence that conditions conducive to gametophyte competition were far from unusual despite a low M/O ratio.

A close relationship was found in this study between microgametophyte numbers and number of seeds produced, and consideration of the slopes of the seeds/microgametophytes regressions leads to a projected requirement of ~14 pollen tubes for full seed set of individual carpels. For all populations and years combined, 21% of styles had >14 microgametophytes, hence an excess over the maximum number required for full seed set. For gametophyte competition to occur, a fairly synchronous arrival and germination of pollen grains is also necessary (Snow, 1986; Bertin, 1990; Spira et al., 1992). Both of these conditions most likely apply generally in *H. foetidus*, because (1) average pollinator visitation rates are so low in my study populations (Herrera et al., 2001) that the time between consecutive pollinator visits to the same flower is expected to range from many hours to several days, hence pollen grains will generally arrive as temporally distinct, widely separated cohorts; (2) only 12 h elapse between pollen deposition and pollen tubes reaching the ovules (Vesprini and Pacini, 2000); and (3) stigma receptivity generally ceases 1 d after pollination (C. M. Herrera, personal observation). It may then be concluded that, despite an average M/O $<$ 1, broad variability around this mean generates conditions conducive to gametophyte competition in about one-fifth of *H. foetidus* styles.

Spatial structures of variance in microgametophyte numbers similar to that documented here for *H. foetidus* have also been reported in a few previous investigations (Levin, 1990; Niesenbaum, 1994; Plitmann and Levin, 1996). The present study also found a significant temporal component superimposed on a fine-grained spatial mosaic, such that differences between populations and the spatial apportionment of regional variance fluctuated between years. On one side, these findings support the conclusion, already anticipated by Snow (1986) for *Epilobium canum*, that the frequency of occurrence of gametophyte competition in nature will ordinarily vary in complex ways between years, regions, populations, individual plants, flowers on the same plant, and even carpels within the same gynoecium. Simple assessments based on short-term studies of one or a few populations should therefore be abandoned in favor of more extensive sampling of natural ranges of variation. In addition to this practical corollary, the particular structure of spatial variability in microgametophyte numbers found here for *H. foetidus*, whereby within-plant variation is the predominant component of variance in local populations (see also Niesenbaum, 1994), suggests few opportunities for selective scenarios derived from gametophyte competition and nonrandom fertilization in this species. Even if local pollen donors differed in pollen-tube growth rate and seed-siring ability and their differences remained consistent across maternal parents (Snow and Spira, 1996; Marshall, 1998), marked stochasticity among and within maternal parents in the characteristics of the competitive environments found within styles would lead to: (1) a considerable reduction in the opportunities of selection among pollen donors based on pollen performance and (2) the ''protection'' from the eroding action of natural selection of additive genetic variation underlying differences in male competitive ability. This would provide a parsimonious explanation for the apparent paradox, noted by some authors (Snow and Spira, 1996; Delph and Havens, 1998), of long-term persistence in natural populations of broad individual variation in pollen competitive ability despite consistent individual differences in male fitness. This example, along with other findings of this study, provide support to the claims of authors that have so far emphasized (although apparently with little success) that empirical field data are an indispensable complement

to manipulative experiments if we are to understand the ecological and evolutionary roles of gametophyte competition and, more generally, sexual selection in natural plant populations (Snow, 1986; Levin, 1990; Niesenbaum, 1994; Plitmann and Levin, 1996).

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