Ecological Context of Breeding System Variation: Sex, Size and Pollination in a (Predominantly) Gynodioecious Shrub

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INTRODUCTION

Most angiosperms are hermaphroditic, which is the ancestral condition from which all other reproductive systems have apparently evolved (Barrett, 2002). The evolutionary pathway from hermaphroditism to dioecy via gynodioecy, a state where hermaphrodite and female individuals co-exist within populations, is theoretically and empirically well supported (Charlesworth, 1999; Webb, 1999; Weiblen et al., 2000). In contrast, the ecological contexts favouring or hindering the two required transitions, namely the spreading of female mutants into co-sexual populations and selection for increased male function in hermaphrodites, are still poorly understood (Ashman, 2006). Indeed, gynodioecy can also be a stable polymorphic breeding system favoured by most frequent inheritance mode that involves several cytoplasmic male sterility (CMS) genes and specific corresponding nuclear restorer alleles for male fertility (e.g. Bailey et al., 2003), and metapopulation structure (Pannell, 1997). This study, based on an ample geographical survey encompassing contrasting ecological scenarios, was designed to evaluate the role of ecological factors in the evolution and maintenance of gynodioecy. Both monomorphic (i.e. hermaphroditic) and gynodioecious populations of Daphne laureola occur within the Iberian Peninsula and, in gynodioecious populations, the proportion of females fluctuates widely, thus providing an exceptional model system.

To spread and persist within populations, female individuals of gynodioecious plants must have some consistent fecundity advantage that compensates for their gametic disadvantage, i.e. for the fact that they are not producing pollen. Such advantage may be achieved by resource compensation due to the reduced costs of their usually smaller and non-polleniferous flowers (Obeso, 2002; for a review, see Shykoff et al., 2003) and/or decreased inbreeding when self-fertilization of hermaphrodites is frequent and inbreeding depression strong (Lewis, 1941; Charlesworth and Charlesworth, 1978). Previous studies on D. laureola have shown that spontaneous autogamy is extraordinarily infrequent among hermaphrodites (Alonso and Herrera, 2001). However, pollinator behaviour promotes geitonogamy in hermaphrodites, and consequently their seed progeny are characterized by extremely low outcrossing rates and considerable inbreeding depression in southern Spanish populations (Alonso, 2004; Medrano et al., 2005).

Female frequency may also depend on environmental conditions and habitat quality (Delph and Carroll, 2001;
Vaughton and Ramsey, 2004). In addition, frequency-dependent selection and pollen limitation of seed production may also act to limit the increase of female frequency beyond a certain threshold (McCauley and Taylor, 1997). In animal-pollinated gynodioecious plants, seed set, pollen limitation and selfing rate are largely determined by pollinator service (Eckhart, 1991; Sakai and Weller, 1999; Ashman, 2000, Williams et al., 2000; Case and Barrett, 2004). Interestingly, at a local scale, the seed production of *D. laureola* was pollen limited only in populations with >50% of females. However, in these populations, pollination success was relatively high due to the characteristics of the habitats (i.e. lower altitude and more favourable conditions for pollinator service of this winter-flowering species; Alonso, 2005). Further studies of intraspecific variation in reproductive characteristics and the relative pollination success of the two sexual morphs along wide geographical and ecological gradients are expected to contribute significantly to understanding the role played by the environment and, more specifically, pollinators in the evolution and maintenance of gynodioecy. Such large-scale studies provide critical links between the micro- and macroevolutionary components of breeding system evolution (e.g. Barrett, 1995; Barrett et al., 2001; Herrera et al., 2006), yet this aspect remains relatively unexplored in gynodioecious plants (but see, for example, Delph, 1990; Wolfe and Shmida, 1997; Asikainen and Mutikainen, 2003; Case and Barrett, 2004; Nilsson and Agren, 2006; Ramsey et al., 2006).

One objective of the present investigation was to determine whether geographical variation in abiotic conditions is predictably related to variation in population breeding system and sex ratio. Such a relationship would be expected from hypotheses relating gynodioecy to environmental adversity (Ashman, 2006) and partially supported by previous studies on *D. laureola* conducted locally along an altitudinal gradient (Alonso and Herrera, 2001). Furthermore, breeding system variation is often correlated with variation in plant growth form and floral display size across species (Vamosi et al., 2003). Thus, a second objective of this study was to test if there are significant correlations within a species between geographical variation in plant size, floral display size and pollination success on one side, and individual sex and population sex ratio on the other. Mean stigmatic pollen load, mean number of pollen tubes developed into the style, and the proportion of flowers without pollen tubes were used as surrogates for pollination success (Alonso, 2005). Finally, particular emphasis was given to the comparison between pollination success of hermaphrodite plants in monomorphic and dimorphic populations because the spread of male sterility mutations within populations would eventually depend on the mating system of hermaphrodites. The hermaphrodite individuals of monomorphic populations are expected to have higher maternal pollination success than hermaphrodites in gynodioecious populations, in which they would eventually reproduce mostly as males (e.g. Case and Barrett, 2004). If inbreeding avoidance is the main force maintaining gynodioecy, as suggested by recent studies on southern Spanish *D. laureola* populations (Medrano et al., 2005) and by those on other species (Ramsey et al., 2006), lower selfing rates and/or environmental conditions in which inbreeding is less detrimental should occur in monomorphic populations.

Specifically, the following questions were addressed. (a) Are population breeding system and female frequency related to abiotic conditions at the broad geographic scale considered? (b) In gynodioecious populations, do female individuals consistently outperform hermaphrodites in flower production and pollination success so as to obtain a higher maternal fitness? (c) Is the magnitude of the female seed production advantage contingent on population environmental conditions and/or female frequency? (d) Do hermaphrodite individuals experience higher maternal fitness in hermaphroditic than in gynodioecious populations?

**MATERIALS AND METHODS**

**Study species**

*Daphne laureola* L. (Thymelaeaceae) is an early-flowering, evergreen shrub growing in the undergrowth of shady mountain forests. It has a Palaearctic distribution ranging from the Atlas Mountains, in Morocco, northwards to England and Hungary (Brickell and Mathew, 1976). In the Iberian Peninsula, the species is abundant in the central and western Cantabrian Range, the Pyrenean Mountains and the southern Betic Ranges. It is infrequent in the eastern Cantabrian Range, and absent from the central Iberian Ranges (Fig. 1). The southeastern Spanish populations of *D. laureola* consist of a mixture of hermaphrodite and female individuals, like other species in its genus (Kikuzawa, 1989). In this region, female frequency is highly variable (3.8–64.3%; 94 populations) and decreases with elevation (Alonso and Herrera, 2001). The genetic basis of gender inheritance in this species is currently unknown. *Daphne laureola* has a characteristic architecture, branches exhibit strongly monopodial growth and leaves occur only at the distal end of stems, where they form a well-defined rosette. The small, tubular, green-yellowish flowers are aggregated into compact axillary inflorescences, and open synchronously in late winter. Individual flowers of both sexes have a single ovule. Female flowers have vestigial stamens that do not produce pollen, and have shorter corolla tubes (mean ± s.d.: 4.9 ± 0.45 mm, n = 45 flowers) than the perfect flowers of hermaphrodites (8.1 ± 0.92 mm, n = 56). Hermaphroditic plants are fully self-compatible. However, fruit production requires flower visitation by pollinators, and an excess of self-pollen may clog up the stigma and reduce fruit set (Alonso and Herrera, 2001). The pollen beetle *Meligethes elongatus* Rosenhauer (Coleoptera: Nitidulidae), small solitary bees and noctuid moths are the main pollinators in the southeastern Spanish populations (Alonso, 2004).

**Study area and sex ratio**

The Iberian Peninsula is extraordinarily heterogeneous in terms of climate, topography and biogeographical
Fig. 1. The locations of the 38 Daphne laureola populations surveyed for sex ratio at the Iberian Peninsula. Population #32 (Roblehondo) is the only one represented here that is located within the Natural Park of Sierras de Cazorla, Segura y Las Villas, where previous studies of altitudinal variation in sex ratio referred to in the text were conducted. White areas of the pie chart graphs indicate the percentage of females in every study population. Fully hermaphroditic populations were not found in the NW region. For reference, the grey shading shows the species distribution range in the Iberian Peninsula.

background, which leads to a considerable diversity of environmental conditions, habitat types and plant communities (e.g. Capel Molina, 1981; Peinado Lorca and Rivas-Martínez, 1987). The southern and central, Mediterranean-climate regions are characterized by evergreen sclerophyllous and sub-sclerophyllous woodlands, dominated by holm or cork oaks (Quercus ilex, Q. suber), frequently mixed with pines (Pinus halepensis, P. nigra, P. pinaster, P. sylvestris), and occasionally with pinsapo fir (Abies pinsapo). In contrast, natural vegetation of the northern Cantabrian and Pyrenean regions is characterized by Atlantic forests dominated by beech (Fagus sylvatica), pines, silver fir (A. alba) and deciduous oaks (Quercus robur, Q. petraea, Q. pyrenaica) [see, for example, Bellot (1978) for a thorough description of the Iberian vegetation mosaic].

During 2003–2005 a total of 38 Iberian populations of D. laureola were visited during the flowering season. The proportion of female and hermaphrodite individuals was estimated at each site by checking the sexual expression of all, or at least 100, individuals per site (Fig. 1). Because the main objective of this study was to assess the possible ecological correlates of breeding system variation, particular effort was made to sample localities throughout the whole range of habitat types, ecological conditions and geographical regions where the species is known to occur in the Iberian Peninsula (Fig. 1). Sampling gaps in Fig. 1 mainly denote zones where the species is absent or very rare. In this study, three wide geographical regions were recognized for the analyses based on major differences in latitude and longitude and sampling discontinuities (northeast, northwest and south, hereafter NE, NW and S, respectively). The abundance of D. laureola is very low between the NE and NW, which provides justification for considering these separately for the analyses.

Pollination success

At the end of the 2003 flowering period, 12 flowers were collected per plant from a sub-set of individuals at 18 study sites widely distributed and differing markedly in female frequency, even though all totally hermaphroditic populations were located in the NE region because the S ones were discovered later on (see Table 1 for locations and number of individuals sampled). Flowers were preserved in FAA solution (2.5 % formaldehyde, 2.5 % acetic acid, 95 % ethanol) until dissection and measurement. Styles were softened in 1 N KOH at 65 °C for 20 min, rinsed with distilled water and stained for 20 min at 65 °C in decolorized aniline blue. The number of pollen grains on the stigma and the number of pollen tubes developed into the style were counted under an epifluorescence microscope for a total of n = 2282 flowers. The pollination success of individual plants was estimated using three different but complementary variables (Alonso, 2005): mean stigmatic pollen loads, which estimated cumulative flower visitation; mean number of pollen tubes per ovule, which reflected the likelihood of microgametophyte competition; and the proportion of flowers within a plant without pollen tubes, which provided an index of the frequency of pollination failure.

Plant and floral display sizes

A more detailed study was conducted in ten northern populations during the 2004 flowering season. Particular effort was invested in the NE region where the highest variation in plant architecture and sex ratio is found. At each site, 40 individuals (20 females and 20 hermaphrodites when available) were randomly selected and plant size was estimated as the total number of leaf whorls. Three partial measures of floral display were also obtained for each plant: proportion of stems bearing flowers; number of inflorescences/stem (based on counts of three stems per plant); and number of flowers/inflorescence (counted in one inflorescence per sampled stem). Total flower production per plant was then estimated by multiplying the number of inflorescences/stem × average number of flowers/inflorescence × number of flowering stems. Similar data for the southern populations are reported in Alonso and Herrera (2001).

Data analyses

All statistical analyses were performed using the SAS statistical package (SAS Institute, 2002). Due to strong heterogeneity of variances among the regions, regional differences in female frequency were analysed by means of an exact-probability median test. Differences between
sexes and among regions in pollination success, plant size and floral display size were analysed by generalized linear mixed models that allowed the effects of fixed and random factors on response variables that do not fit to normal distribution of errors to be analysed simultaneously (Procedure Glimmix in SAS 9.13; Littell et al., 1996).

Negative binomial error distribution was used for count data (stigmatic pollen load, number of pollen tubes, number of stems, number of inflorescences/stem, total flower production), binomial error distribution for the proportion of unfertilized flowers and normal error distribution for the average number of flowers/inflorescence. A single analysis for each response variable tested simultaneously for the effect of region and sex, considering both effects as fixed and defining populations as random. In particular, differences between sexes were analysed as a priori contrasts testing for differences between female and hermaphrodite plants in gynodioecious populations, and between hermaphrodite plants growing in gynodioecious and hermaphrodite populations. Data obtained from multiple flowers per plant were averaged, and subsequent analyses were based on the individual means. Unless otherwise stated, least-squares means (+ s.e.) that estimate the marginal means corresponding to the fixed effects over a balanced population will be shown. Correlations of population means with sex ratio and local temperature and precipitation predicted by interpolation models based on 30 years of meteorological data (Bustamante, 2003) were calculated by the Spearman rank coefficient.

### RESULTS

#### Geographical variation in sex ratio

Not all *D. laureola* populations in the Iberian Peninsula are gynodioecious. Female frequency ranged from zero, i.e. totally hermaphroditic populations, to >45% in some

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**Table 1. Average estimates of pollination success in the 18 Daphne laureola Iberian populations sampled in spring 2003 (s.d. in parentheses; n = 12 flowers per plant). For population number and location see Fig. 1.**

<table>
<thead>
<tr>
<th>Region</th>
<th>Population number (% females)</th>
<th>Sex*</th>
<th>No. of plants</th>
<th>No. of pollen grains</th>
<th>No. of pollen tubes</th>
<th>% unfertilized flowers</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>29 Herm</td>
<td>6</td>
<td>145.0 (200.2)</td>
<td>16.5 (11.8)</td>
<td>15.3 (33.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 Herm</td>
<td>10</td>
<td>106.5 (256.2)</td>
<td>20.1 (44.3)</td>
<td>25.0 (75.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27 Herm</td>
<td>10</td>
<td>85.6 (115.3)</td>
<td>14.5 (27.6)</td>
<td>30.8 (49.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26 Herm</td>
<td>10</td>
<td>143.7 (122.2)</td>
<td>19.1 (23.4)</td>
<td>16.7 (47.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 Herm</td>
<td>6</td>
<td>155.0 (222.5)</td>
<td>20.1 (19.3)</td>
<td>15.1 (38.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 Herm</td>
<td>10</td>
<td>202.2 (192.0)</td>
<td>25.7 (20.0)</td>
<td>9.2 (37.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 Herm</td>
<td>6</td>
<td>166.9 (127.2)</td>
<td>25.9 (14.8)</td>
<td>8.6 (31.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18 Herm</td>
<td>9</td>
<td>112.7 (133.4)</td>
<td>21.0 (22.8)</td>
<td>16.5 (48.3)</td>
<td></td>
</tr>
<tr>
<td>NW</td>
<td>19 (7.8%) Fem</td>
<td>4</td>
<td>9.5 (48.3)</td>
<td>1.1 (6.5)</td>
<td>83.3 (66.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 (44.0%) Fem</td>
<td>9</td>
<td>103.7 (123.1)</td>
<td>20.2 (31.5)</td>
<td>25.9 (71.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 (54.0%) Fem</td>
<td>7</td>
<td>6.2 (13.9)</td>
<td>1.1 (5.4)</td>
<td>77.4 (77.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (41.7%) Fem</td>
<td>5</td>
<td>76.6 (51.3)</td>
<td>3.4 (10.5)</td>
<td>42.4 (86.9)</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>36 (13.2%) Fem</td>
<td>6</td>
<td>11.0 (22.2)</td>
<td>4.4 (6.5)</td>
<td>35.6 (79.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35 (17.1%) Herm</td>
<td>6</td>
<td>93.3 (97.9)</td>
<td>14.1 (18.7)</td>
<td>8.3 (25.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33 (45.0%) Fem</td>
<td>6</td>
<td>15.4 (36.3)</td>
<td>5.8 (15.2)</td>
<td>50.0 (104.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32 (34.5%) Herm</td>
<td>6</td>
<td>115.1 (230.2)</td>
<td>12.1 (15.4)</td>
<td>20.8 (83.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31 (32.3%) Fem</td>
<td>6</td>
<td>18.9 (147.5)</td>
<td>16.2 (15.9)</td>
<td>9.7 (21.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 (40.0%) Fem</td>
<td>6</td>
<td>12.5 (146.2)</td>
<td>23.2 (21.2)</td>
<td>62.5 (101.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29 (40.0%) Fem</td>
<td>6</td>
<td>10.8 (147.5)</td>
<td>23.2 (21.2)</td>
<td>62.5 (101.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 (40.0%) Fem</td>
<td>6</td>
<td>89.4 (212.3)</td>
<td>13.9 (19.1)</td>
<td>11.1 (35.0)</td>
<td></td>
</tr>
</tbody>
</table>

* Herm, hermaphrodite individuals; Fem, female individuals.
gynoecious populations (Fig. 1). The observed variation in female proportion was not homogeneously distributed among regions (χ² = 10.55, P = 0.005; exact-probability median test). NW populations have consistently more females (mean ± s.d. = 39.6 ± 6.1%; median = 40.6%; n = 12 populations) than those in the NE (23.3 ± 24.5%; median = 7.8%; n = 17) and S (19.67 ± 15.6%; median = 17.2%; n = 9).

In the two northern regions, female proportion tended to decrease from west to east (Fig. 1; r = -0.37, n = 29, P = 0.045); the longitudinal location was not significantly correlated to mean annual temperature (r = -0.15, n = 29, P = 0.45) but correlated negatively to precipitation (r = -0.44, n = 29, P = 0.02). Female proportion correlated negatively with mean annual temperature (r = -0.60, n = 29, P = 0.0006; Fig. 2), and positively with the total annual precipitation (r = 0.62, n = 29, P = 0.0003) that ranged between 635-6 and 2093 mm. Congruently, the hermaphroditic populations were characterized by significantly higher temperatures (10.9 ± 0.6°C vs. 7.6 ± 0.4°C; F1,27 = 23.46, P < 0.0001) and lower precipitation levels (726.5 ± 106.7 mm vs. 1320.0 ± 56.1 mm; F1,27 = 52.6, P < 0.0001) were similar to those observed in the whole data. Since the NE region is a geographically more restricted area, where both monomorphic and dimorphic populations were equally represented in the sample (Fig. 1), these results suggest that the differences between the hermaphroditic and gynoecious populations were not likely to be artefacts due to spatial autocorrelation.

In the southern region, the percentage of females decreased from east to west (r = -0.77, n = 9, P = 0.013). Similarly to northern populations, the percentage of females was inversely related to mean local annual temperature (r = -0.94, n = 9, P = 0.0002; Fig. 2), and hermaphroditic populations were associated with higher temperatures than gynoecious ones (16.4 ± 0.6°C vs. 11.5 ± 0.3°C; F1,7 = 53.11, P = 0.0002). In contrast, female frequency was not significantly related to local precipitation level (r = -0.34, n = 9, P = 0.37), that was much lower than in northern sites and ranged between 470.5 and 1270 mm.

**Plant and floral display sizes**

Adult plants of *D. laureola* vary widely in size and architecture across the Iberian Peninsula, ranging from slim single-stemmed individuals to large shrubs consisting of >400 stems. Data collected in the ten northern populations studied more intensively (see Supplementary Information available online for study sites and sample sizes) reveal that plants in the NE were smaller (F1,388 = 10.38, P = 0.0014; Fig. 3A), and had fewer flowering stems (F1,356 = 123.75, P < 0.0001; one population excluded, see Supplementary Information for details) than in NW populations. However, the mean numbers of inflorescences/stem and flowers/inflorescence were similar between these two regions (P > 0.20 in both cases). Regional differences in floral display size are confirmed when all the reproductive parameters measured are combined multiplicatively to estimate total flower production per individual (F1,388 = 6.23, P = 0.013). Total flower production per plant was considerably lower in the NE (Fig. 3B; median = 54 flowers/plant) than in the NW populations (median = 967 flowers/plant).

As regards differences between sexes in gynoecious populations, it was found that female plants were significantly larger than hermaphrodites (21.4 ± 6.9 vs. 13.4 ± 4.3 stems; F1,388 = 10.38, P = 0.0014), and they also tended to have more flowering stems, although the difference was only marginally significant (15.5 ± 2.2 vs. 10.9 ± 1.4 stems; F1,356 = 3.45, P = 0.06). Mean number of inflorescences/stem and the average number of flowers/inflorescence did not differ between the sexes (P > 0.20; see Supplementary Information for details). Altogether, female individuals produced more flowers than hermaphrodites in the gynoecious populations (638.9 ± 286.2 vs. 414.8 ± 182.8 flowers; F1,388 = 3.85, P = 0.05).

Finally, the size of hermaphrodite plants growing in hermaphroditic and gynoecious populations did not differ significantly (8.6 ± 4.3 vs. 13.4 ± 4.3 stems;
Not provided.
Intraspecific variation in female frequency is a widespread phenomenon in gynodioecious species (Webb, 1999). In species with both monomorphic and dimorphic populations, gynodioecy seems to be associated with harsh dry environments (Vaughton and Ramsey, 2004; Ashman, 2006 and references therein). However, gynodioecious populations of *D. laureola* were found at sites characterized by higher precipitation and lower temperatures than purely hermaphroditic populations; this pattern occurred consistently both at the scale of the whole Iberian Peninsula and within the NE region, where both monomorphic and dimorphic populations were relatively common. Indeed, hermaphroditic populations in this region were found in relatively isolated coastal and pre-Pyrenean mountains with Mediterranean climate, whereas gynodioecious populations were predominantly located in Atlantic-climate environments. Furthermore, female frequency was inversely related to local temperature (Fig. 2), and in the two northern regions it increased with annual precipitation. These results contrast with the negative relationship between female frequency and site elevation observed in a southeastern Mediterranean mountainous region (Alonso and Herrera, 2001), that was consistent with the expectation of a higher female frequency in harsher environments. The contrasting results obtained by these two investigations conducted at different spatial scales suggest that different selective and stochastic processes might prevail at different geographic scales (see also Nilsson and Agren, 2006). In the following paragraphs there follows a discussion on how the selective pollination context might influence intraspecific geographic variation in the breeding system of *D. laureola*.

**Plant and floral display sizes**

Plants of *D. laureola* growing in different regions of the Iberian Peninsula differed mainly in their size. Plants growing in the NE region had fewer than six leaf whorls on average, with just 3–5 stems bearing flowers, and were thus considerably smaller than plants growing in the NW (Fig. 3A) and also in comparison with S populations in which average individual size ranged between 34 and 480 leaf whorls per plant (*n* = 8 populations; C. Alonso, unpubl. data).

The main consequence of plant size variation in terms of sexual reproduction was related to flower production, that was estimated to vary on average between 15 and 3978 flowers per plant among the populations. Variation had a strong regional component, as plants in the NE region produced 8.6- and 13.5-fold fewer flowers than those in the NW (Fig. 3B) and S regions (see Alonso and Herrera, 2001), respectively. Such huge difference in flower production in a highly synchronously flowering species should have consequences for the plant–pollinator interaction, since the size of floral display also influences pollinator attraction and behaviour in gynodioecious species (e.g. Eckhart, 1991; Ashman and King, 2005).

Geitonogamy, the transfer of self pollen between flowers within an individual plant, usually increases with floral display (Harder and Barrett, 1995). This relationship may explain why, at the interspecific level, dioecy tends to evolve more frequently in clades with many-flowered inflorescences in some groups (Vamosi et al., 2003). At the intraspecific level, the results for *D. laureola* support a relationship between floral display size and the evolution of gynodioecy. On one hand, previous studies conducted...
on S populations characterized by large floral displays suggested that geitonogamy is frequent and, thus, male sterility would be a mechanism for preventing selfing, particularly advantageous because it is coupled with strong inbreeding depression (Medrano et al., 2005). On the other hand, the NE hermaphroditic populations were characterized by very small floral displays and thus presumably were the ones least susceptible to geitonogamous pollinations. These results suggest that the benefits of male sterility obtained through outbreeding might not be substantial enough to favour a gynodioecious breeding system in populations characterized by small plants and reduced floral display. The observed higher maternal pollination success of hermaphrodites in the monomorphic populations (see below) is consistent with this suggestion.

After statistically accounting for the effects of regional variation, female plants were 1–6 times larger and, as a consequence, produced 1–5 times more flowers than hermaphrodites in the gynodioecious populations. This finding is consistent with the generalized notion that male function is costly and females re-invest the saved resources in modular growth and, subsidiary, flower production (see Eckhart and Seger, 1999); this aspect does not seem to have often gained support from studies on vegetative traits in gynodioecious species (Ashman, 2005). Indeed, the pattern was not upheld in all of the study populations (Supplementary Information available online; see also Alonso and Herrera, 2001), highlighting that the cost of male function and the magnitude of gender dimorphism might also be context dependent (Alonso et al., 2005; Delph and Wolf, 2005).

**Pollination success**

In early blooming species like *D. laureola*, characterized by extraordinarily low pollinator visitation rates (Alonso, 2004), direct estimates of identity, abundance and behaviour of pollinators are impractical at the broad geographic scale of this study. The scarcity of floral visitors was confirmed during the visits to *D. laureola* populations in all the study regions, and supported by the high percentages of unfertilized flowers, which ranged between 31·9 and 83·3 % and 8·3 and 65·3 % in female and hermaphrodite individuals of the gynodioecious populations, respectively, and between 8·6 and 30·8 % in hermaphroditic populations. Pollination success of individual plants was thus estimated using three different, complementary parameters: mean stigmatic pollen loads; mean number of pollen tubes per ovule; and the proportion of unfertilized flowers within a plant. We are confident that these parameters provide a comprehensive description of the ‘pollinating environment’ faced by the *D. laureola* populations studied, and the last is also a good correlate of individual fruit set in females (Alonso, 2005). In a population studied in two other years (Roblehondo site in Alonso, 2005), estimates of pollination success were similar to those of the present study. This suggests yearly consistency in the pollinating environment and that the geographic variation observed in 2003 could be representative of prevailing conditions.

As in other gynodioecious species, differences among the sites in the composition and abundance of the pollinator assemblage, as well as in overall pollen availability resulting from differences in sex ratio, could both contribute to spatial variation in pollination success (e.g. Delph, 1990; Eckhart, 1991; Ashman, 2000; Case and Barrett, 2004). In addition, abiotic conditions and particularly temperature could determine the activity and fertilization effectiveness of insect pollinators (e.g. Totland, 2001). The positive relationship between mean annual temperature and pollination success at the population level observed here (Fig. 5) confirms previous findings suggesting that abiotic conditions determine the reproductive success of this early flowering species more strongly than the sex ratio (Alonso, 2005). Regarding variation in pollinator abundance and species composition, and because of the difficulty in characterizing pollinator assemblages mentioned above, it can only be speculated at present that *Meligethes* pollen beetles seem to be relatively common only in the southern *D. laureola* populations. In these populations, the pollen beetles are found at all altitudes, and occur in both hermaphroditic and gynodioecious populations (Alonso, 2004; and unpubl. data), while they have never been observed during the sampling visits to the northern populations. Along with the higher quantitative estimates of pollination success obtained in southern populations, in which on average only 14 % of ovules were not fertilized at intermediate altitudes compared with the >33 % observed in the northern populations, the present findings suggest that the abundance of pollen beetles is probably a major determinant of seed set in *D. laureola*.

Geographical variation in the quality of pollination, and particularly its correlations with geitonogamy and resulting inbreeding, may be another factor contributing to the observed broad-scale variation in breeding system in gynodioecious species (e.g. Ramsey et al., 2006). Previous studies on *D. laureola* have shown that spontaneous autogamy is extraordinarily infrequent (Alonso and Herrera, 2001), but seed progeny of hermaphrodites are characterized by extremely low outcrossing rates in S gynodioecious populations (Medrano et al. 2005). In the NE hermaphroditic populations, where geitonogamy is expected to be much less frequent due to reduced floral display, individuals received on average 1·5 times more pollen grains and twice as many tubes per pistil than the hermaphrodite individuals in gynodioecious populations. A higher pollen tubes/ovules ratio in hermaphroditic populations would indicate increased quality of the pollen received (Alonso, 2005). Furthermore, the increased opportunity for pollen tube competition would eventually lead to increased off-spring quality and might consequently reduce inbreeding depression (e.g. Lankinen and Armbruster, 2007). Such results support the hypothesis that hermaphrodite individuals of monomorphic populations should have higher maternal fitness in order to preclude the spread of females. It should be noted here that in two of the NE hermaphroditic populations studied, one single female individual was found (see Supplementary Information for details), and thus the male sterility mutation was not totally absent.
from these populations. Reduced floral display might thus contribute to the relative success of hermaphroditism in the NE because it apparently increased pollination quality without reducing the proportion of visited flowers per plant. To test rigorously for the importance of geitonogamy as a determinant of breeding system evolution in this species, the mating system and inbreeding depression should also be estimated in the purely hermaphroditic populations.

Combining the average population estimates of flower production and pollination failure shown above, upper estimates of total seed production per sex and site were obtained. Despite the fact that the females produced more flowers in all of the northern populations studied (see Supplementary Information), their flower production was insufficient to compensate for their much lower proportion of fertilized flowers in three out of five populations. The two exceptions are from the NE region: in population #16 average seed production was estimated as 65.6 seeds/plant in females and 41.8 seeds/plant in hermaphrodites, and in population #19 the corresponding estimates were 70.0 vs. 62.8 seeds/plant. Excluding these two sites, estimates of female seed production relative to hermaphrodites ranged between 0.34 and 0.95. Furthermore, the absence of a significant correlation between female frequency and the proportion of unfertilized flowers of both female and hermaphroditic individuals supports previous findings suggesting that differences between sexes in seed output in *D. laureola* are insensitive to variation in sex ratio (Alonso, 2005; Medrano et al., 2005).

**Conclusions**

The present results highlight the contribution of both abiotic and pollinator service to intraspecific geographic variation in plant breeding systems. Contrary to the most common rule for sub-dioecious species, hermaphroditic populations were located at warmer sites and female percentage decreased with local temperature. The findings do not support the idea that enhanced female seed production through resource compensation, and frequency-dependent selection, are important factors contributing to the maintenance of gynodioecy in *D. laureola* in the Iberian Peninsula. However, inbreeding avoidance coupled with strong inbreeding depression could be crucial factors in some regions where the populations are characterized by large plants and floral displays. This hypothesis, however, does not hold over the large geographic study area. In a few southern populations hermaphroditism was not associated with small plant size and low flower production (C. Alonso, unpubl. data). Ongoing studies on the mating system of these hermaphroditic populations should explain whether this inconsistency might result from a reduced geitonogamy associated with differences in pollinator service and/or a lower inbreeding depression associated with local abiotic conditions.

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**SUPPLEMENTARY INFORMATION**

Supplementary information is available online at http://aob. oxfordjournals.org/ and gives average estimates of plant and floral display sizes recorded in the ten *Daphne laureola* northern populations that were studied.

**LITERATURE CITED**


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