

## Comparative floral and vegetative differentiation between two European *Aquilegia* taxa along a narrow contact zone

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**Abstract.** As a first step in determining the identity and relative importance of the evolutionary forces promoting the speciation process in two closely related European taxa of *Aquilegia*, we investigated the levels of morphological variation in floral and vegetative characters over the narrow region where their ranges enter into contact, and evaluate the relative importance of both types of traits in their differentiation. A total of 12 floral and ten vegetative characters were measured on 375 plants belonging to seven *A. vulgaris* populations and six *A. pyrenaica* subsp. *cazorlensis* populations located in southeastern Spain. Floral and vegetative morphological differentiation occur between taxa and among populations within taxa, but only vegetative characters (particularly plant height and leaf petiole length) contribute significantly to the discrimination between taxa. Differentiation among populations within taxa is mostly explained by variation in floral traits. Consequently, morphological divergence between the two taxa cannot be interpreted as an extension of among-population differences occurring within taxa. Multivariate vegetative, but not floral, similarity between populations could be predicted from geographical distance. Moreover, the key role of certain vegetative traits in the differentiation of *A. vulgaris* and *A. p. cazorlensis* could possibly be attributable to the contrasting habitat requirements and stress tolerance strategies of the two taxa. These preliminary findings seem to disagree with the

currently established view of the radiation process in the genus *Aquilegia* in North America, where the differentiation of floral traits seems to have played a more important role.

**Key words:** *Aquilegia pyrenaica* subsp. *cazorlensis*, *Aquilegia vulgaris* subsp. *vulgaris*, floral character, Iberian Peninsula, inter- and intraspecific variation, morphological differentiation, Ranunculaceae, vegetative character.

The radiation of angiosperms has been tightly linked to the diversification in floral morphology and function, and this effect is particularly pronounced in animal-pollinated lineages (Grimaldi 1999, Fenster et al. 2004, but see Waser 1998, Gorelick 2001). Specifically, floral characters contribute to species separation much more often among animal-pollinated taxa than among wind- and water-pollinated taxa (Grant 1949). Progress in our understanding of floral diversification has been mainly based on examination of phylogenetic and ecological correlates of floral variation at the species level and above (e.g. Barrett and Graham 1997, Hapeman and Inoue 1997, Hodges 1997, von Hagen and Kadereit 2003, Graham and Barrett 2004) and studies of phenotypic selection at the within-population

level based on either naturally-occurring (e.g. Campbell et al. 1991, Herrera 1993, Gómez 2000, Maad 2000) or artificially induced (e.g. Herrera 2001, Aigner 2004, Castellanos et al. 2004) variation in floral traits. In contrast, there have been relatively few investigations focusing on the connections between micro- and macroevolutionary patterns of floral variation (Miller 1981, Barrett 1995, Johnson 1997), and on intraspecific floral variation and its relationship to evolutionary divergence in pollinators (see however e.g. Herrera 1990, Robertson and Wyatt 1990, Arroyo and Dafni 1995, Boyd 2002, Herrera et al. 2002, Silva-Montellano and Eguiarte 2003).

Factors other than pollinators may also promote floral divergence (see Wilson and Thomson 1996 and references therein) or account for population differences in floral characteristics along the geographical range of a species (e.g. Galen 1999 and references therein). Variation in abiotic factors, e.g. soil nutrient availability or water stress, may ultimately explain population differences in the floral characteristics of some species, either directly by selection on certain floral characteristics (Frazee and Marquis 1994, Galen et al. 1999, Galen 2000) or indirectly through their influence on vegetative characters allometrically or pleiotropically linked to floral traits (Bond and Midgley 1988; Midgley and Bond 1989; Andersson 1993, 1997). Studying geographical variation on floral characters in conjunction with vegetative traits should provide a more reliable evaluation of the relative importance of pollinators in the divergence process that could be taking place within a species.

The main goal of this paper is to compare the levels of phenotypic morphological variation in floral and vegetative characters among populations of two closely-related European taxa of the genus *Aquilegia*, *A. vulgaris* subsp. *vulgaris* (*A. vulgaris* hereafter) and *A. pyrenaica* subsp. *cazorlensis* (*A. p. cazorlensis* hereafter), throughout a region where their ranges enter into close contact, as a first step in determining the identity and relative importance of the evolutionary forces

promoting their differentiation. North American species of *Aquilegia* (Ranunculaceae) have become the textbook example of adaptive radiation driven by specialization on different pollinators in combination with ecological niche diversification (Schluter 2000 and references therein). In Europe, the genus *Aquilegia* comprises roughly the same number of species as in North America (Munz 1946, Jalas et al. 1999), yet the factors underlying their diversification remain essentially unexplored to date. This study was conducted along the single southeastern mountain of the Iberian Peninsula where populations of the widely distributed *A. vulgaris* coexist with the narrow endemic *A. p. cazorlensis*. In this region, the two taxa are pollinated by the same few bumblebee species (see below), though they grow in locally different habitats. In particular, given that *A. p. cazorlensis* occurs in more stressful sites (such as rock crevices and sandy soils around cliff bases) than *A. vulgaris*, we expect that the role of certain vegetative traits (particularly those related to plant or leaf size) should be particularly important in their differentiation, as found for other pairs of narrow endemic and widespread closely related taxa (Lavergne et al. 2003, 2004). Specifically, the following questions will be addressed in this paper: (1) Do the two taxa studied exhibit discernible geographic variation in floral and vegetative traits at the relatively small spatial scale of this investigation? If they do, (2) which type of traits, floral or vegetative, are those most intraspecifically variable? (3) Do floral and vegetative traits vary in unison across populations of the same species? (4) Can interspecific differences in the traits examined be interpreted as extensions of among-population differences occurring within species? And (5) does the observed variation conform to any discernible geographical pattern?

## Materials and methods

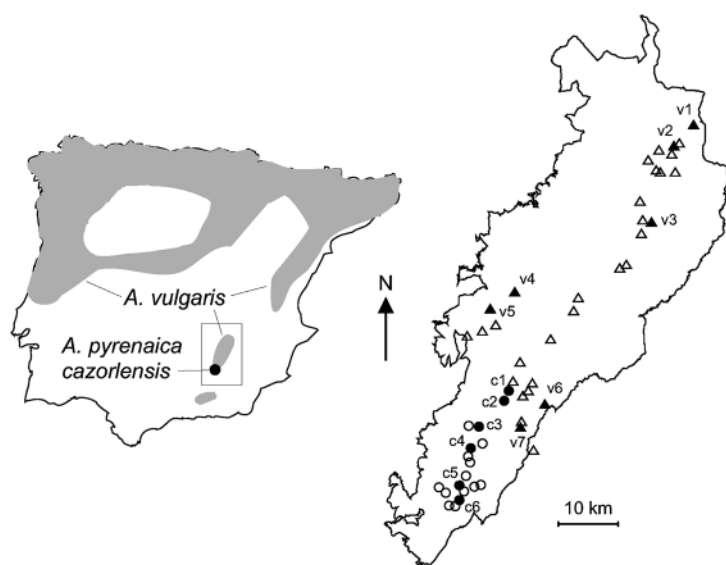
**Study species and sites.** *Aquilegia vulgaris* L. is a widely distributed perennial herb whose natural

populations are common throughout Eurasian mountain forests, although it sometimes occurs in open woodlands and meadows at or around sea level. In our study sites plants grow along stream margins or poorly drained open meadows around springs at 900 to 1700 m of elevation. Flowering takes place mainly from May to early June. *Aquilegia pyrenaica* subsp. *cazorlensis* (Heywood) Galiano and Rivas-Martínez is a narrowly endemic perennial herb, restricted to a few populations in the Sierras de Cazorla and El Pozo, in the Spanish province of Jaén (Fig. 1), occurring from 1200 to 1950 m of altitude. Plants grow in rifts of limestone outcrops and on sandy soils in shady, damp sites at cliff bases, and bloom during June-early July.

In both species, a mature plant consists of a slender rhizomatous stem with one to several basal rosettes, each with 3–6 pubescent ternate compound leaves. Mature plants can produce one to several paniculate inflorescences, each bearing 1–13 (*A. vulgaris*) or 1–8 (*A. p. cazorlensis*) flowers, although not all mature individuals flower in any given year. Flowers of both species range from pale blue to purple, are pendant and radially symmetrical, with five petaloid sepals alternating with five petals elongated into nectar-producing spurs. The

flowers are bisexual, self-compatible and to some extent self-pollinating in the absence of pollinators (unpublished data). In preliminary pollinator censuses and observations, the predominant visitors to both *Aquilegia* species at the study region are bumblebees (Table 1), though a variety of other insects visit the flowers occasionally.

This study was conducted during May-June 2004 on seven *A. vulgaris* populations and six *A. p. cazorlensis* populations located in the Natural Park Sierras de Cazorla-Segura-Las Villas, in southeastern Spain, where both species coexist (Fig. 1). The 13 study populations were regularly scattered over the known distribution of each taxon in the region (Fig. 1) and were located along a latitudinal gradient. While the region has a Mediterranean-type climate characterized by a marked summer drought, with 90% of the annual precipitation falling during October-April, within the Natural Park a significant negative correlation between latitude and yearly total rainfall exists (Herrera et al. 2006). Given the geographic proximity of some populations of both taxa in this area (Fig. 1) and that hybridization is frequent in this genus (Munz 1946), hybridization between *A. vulgaris* and *A. p. cazorlensis* could occur or have occurred in some



**Fig. 1.** Distribution of *Aquilegia vulgaris* and *Aquilegia pyrenaica* subsp. *cazorlensis* in the Iberian Peninsula. The map on the right shows the location of the Natural Park Sierras de Cazorla-Segura-Las Villas in the Iberian Peninsula, and known populations of *A. vulgaris* (triangles) and *A. p. cazorlensis* (circles) in the study region. Filled symbols are the study populations: v1, Río Tus; v2, Cascada del Saltador; v3, Barranco de los Jabalises; v4, Cuevas del Peinero; v5, Aguascebas de Gil Cobos; v6, La Cabrilla; v7, Barranco del Guadalentín; c1, Covacho del Aire; c2, La Charca; c3, La Mesa; c4, Cerrada del Pintor; c5, Cabañas; c6, La Canal

**Table 1.** Preliminary information on floral visitors to *Aquilegia vulgaris* and *A. pyrenaica* subsp. *cazorlensis* at the Sierras de Cazorla-Segura-Las Villas study region. The table shows the number of flowers visited by three *Bombus* species and other insects. Most visits were recorded during 1h-long pollinator censuses in 6 populations of each species during peak bloom, but include also casual observations while working with the plants. In total, we spent 6–8 whole days in the field with each species while in bloom

	<i>A. vulgaris</i>	<i>A. p. cazorlensis</i>
<i>Bombus pascuorum</i>	41	25
<i>Bombus pratorum</i>	45	23
<i>Bombus terrestris</i>	0	5
Other visitors <sup>a</sup>	9	34 <sup>b</sup>

<sup>a</sup> Include visits by other bees (*Lasioglossum* spp., other Halictids, *Andrena* sp., *Xylocopa* sp., *Eucera* sp.), syrphid flies (*Eristalis tenax*, *Pipiza* sp.), the day-flying moth *Macroglossum stellatarum*, and small Nitidulidae (Coleoptera)

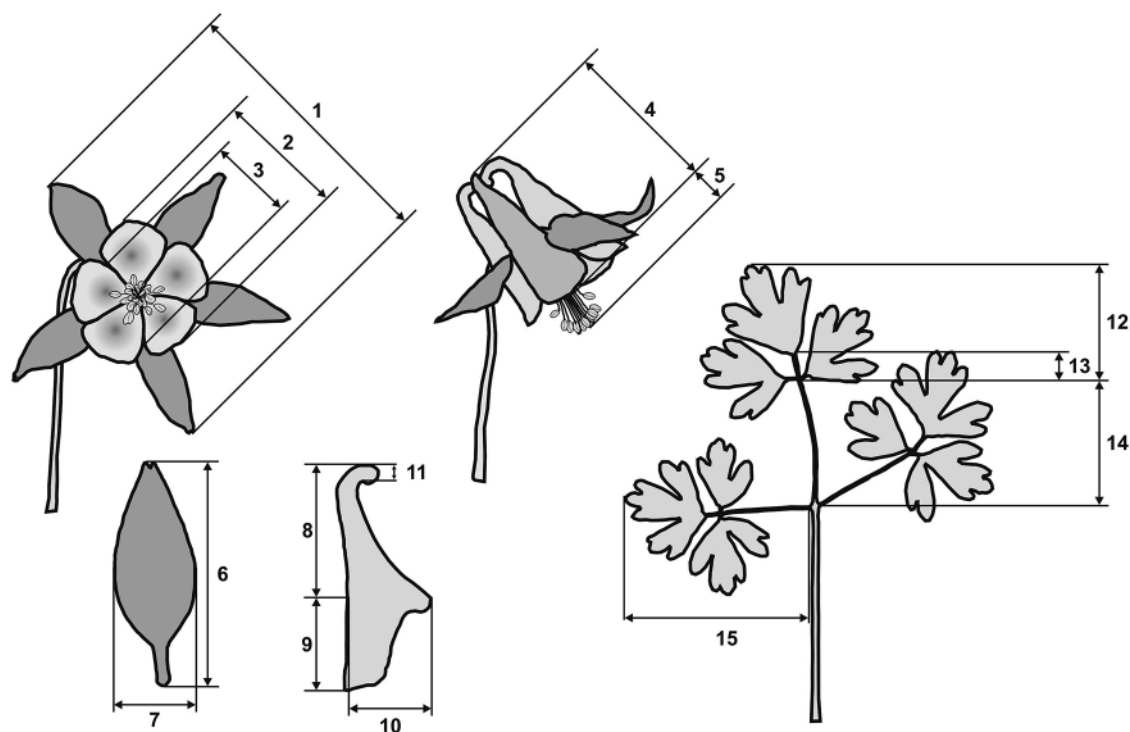
<sup>b</sup> 24 of these visits were recorded in a single population, all by small *Lasioglossum* bees. The bees were collecting pollen and appeared to be making little or no contact with stigmas

populations, yet the phenological differences in flowering period could limit the possibilities of interspecific pollen transfer. In addition, because plants of both species are restricted to humid soils, populations grow apart from each other and are separated by dry areas with sclerophyllous, Mediterranean-type vegetation. Geographical coordinates of each population were recorded with a GPS receiver. Pairwise distances between study sites ranged between 3.7–58.6 km in *A. vulgaris*, and between 1.2–20.4 km in *A. p. cazorlensis*.

**Floral and vegetative measurements.** Between 13 and 32 flowering plants were randomly selected at variable distance intervals (always >0.5 m) along arbitrarily arranged transects within each study population. To avoid phenological differences between individuals, plants that had only just begun to bloom (inflorescences with one or two flowers opened) were chosen. Measurements of floral and vegetative traits of each plant were made from both fresh and field-pressed material. The total number of individuals sampled was 375 (N = 195 for *A. vulgaris* and N = 180 for *A. p. cazorlensis*).

To quantify inter- and intra-specific variation in flower morphology, one newly opened flower was chosen from each of the 375 selected plants and two digital photographs (one frontal and one lateral) were taken in the field with a sticker glued to the flower for scale. Care was taken to sample one of the first opened flowers of each inflorescence that had at least one anther shedding pollen. Before taking the pictures, the flowers were orientated in a standardized way to ensure consistency in

measurements. One sepal and one petal from these flowers were also removed and pressed dry. In the lab, all dried petals and sepals from each population were mounted on a separate sheet of paper with a scale, and digital images were captured using a scanner. All digital images were calibrated and manually analysed using SigmaScan Pro (version 5.0). Eleven floral traits were measured from the digital images for each individual (Fig. 2): calyx diameter, corolla diameter, corolla “fusion” (internal diameter of the corolla, measured between internal adjoining margins of two opposite petals; although *Aquilegia* flowers have free petals, the degree of the physical adhesion of their margins measures level of corolla aperture), petal length, stamen exertion, sepal length and width, spur length, petal blade length, spur aperture (width at its aperture) and spur width above the nectary. Additionally, the degree of spur curvature (not shown in Fig. 2) from each individual was estimated by visual assessment of pressed petals. Each petal was assigned to one of 6 spur curvature subjective categories: 0 for straight spurs, 1 for spurs curved less than 45°, 2 for spurs between 45° and 90°, 3 for spurs forming a 90° angle, 4 for those with more than a 90° angle, and 5 for completely hooked spurs. Some of these floral traits were selected because they describe morphological features potentially influenced by selection from pollinators (e.g. flower size, spur length, stamen exertion), but others (e.g. spur curvature, stamen exertion) were also chosen because they serve as taxonomic criteria to differentiate *Aquilegia* species and subspecies in the Iberian Peninsula (Díaz



**Fig. 2.** Floral and leaf traits considered in this study. Floral traits measured from photographs: **1** Calyx diameter, **2** Corolla diameter (external), **3** Corolla “fusion” (internal corolla diameter, see text for a more detailed description), **4** Petal length, **5** Stamen exsertion. Floral traits measured from pressed sepals and petals: **6** Sepal length, **7** Sepal width, **8** Spur length, **9** Petal blade length, **10** Spur “aperture” (spur width at its aperture), **11** Spur width above nectary. Leaf traits measured from pressed leaves: **12** Length of central pinna, **13** Length of third order petiole, **14** Length of petiolule, **15** Length of lateral leaflet

González 1986). Moreover, this set of measurements includes those that differentiate North American species (Grant 1952).

To quantify variation in vegetative and life history traits, the following measurements were recorded in the field for each focal plant: overall plant height (measured as the height of the tallest inflorescence), number of basal rosettes (care was taken to ensure that all of them belonged to the selected plant by digging it up lightly if necessary), number of leaves per rosette, total number of leaves, and inflorescence size (estimated as the mean number of flowers per inflorescence). One fully expanded leaf per plant was selected and pressed for later morphological analysis. In the lab, the number of pinnulae in each pressed leaf was counted and the sizes of different leaf parts were measured to the nearest 0.01 mm using digital callipers. Leaf traits measured were: length of the central pinna, length of the third order petiole, length of the petiolule and

length of the lateral leaflet (Fig. 2). Width measurements of different leaf blade parts were also taken, but they had to be excluded from analyses because high herbivory levels considerably reduced sample sizes. The 10 measured vegetative traits for each focal plant were selected because of their taxonomical relevance (plant height) or because they could be potentially influenced by abiotic factors (plant and leaf sizes). Although size and height of the inflorescence may also be influential on pollinator foraging, we included these variables among vegetative traits because they mainly reflect differences in plant size and there was no evidence that their variation translated into different pollinator faunas (Table 1).

**Statistical analysis.** As the purpose of this study is to elucidate overall patterns of morphometric variation between taxa and among populations within taxa rather than the patterns of variation in some particular traits, *Aquilegia* taxa and populations were compared from a multivar-

iate perspective, initially including both vegetative and floral traits, but also exploring the separate effects of the two types of traits. Morphological differences between taxa and among populations were first tested by means of multivariate analysis of variance (MANOVA). Two separate models were tested, each with one fixed effect (“taxa” or “population”) and the 12 vegetative and/or 10 floral morphological characters as dependent variables. Next we used Canonical Discriminant Analysis (CDA) to determine which variables best discriminated between populations. Morphological variables were log- or square root-transformed when necessary to correct for heteroscedasticity. CDA derives canonical variates, i.e. linear combinations of the variables that summarize, in this case, between-population variation. The first canonical variate in our analyses (CV1) explained a considerable amount of the variation (see Results), so we used the variance in canonical scores to further investigate the relative roles of vegetative and floral traits in distinguishing populations and taxa. Possible geographical patterns were explored by means of (a) relating the positions of the populations on CV1 to latitude; and (b) using Mantel tests to test for relationships between geographical and morphological distances (computed from coordinates on the first two canonical variates, CV1 and CV2) between populations. MANOVA and CDA were run in Systat, and Mantel tests in Passage 1.1 (Rosenberg 2001). For all other tests we used the SAS package.

## Results

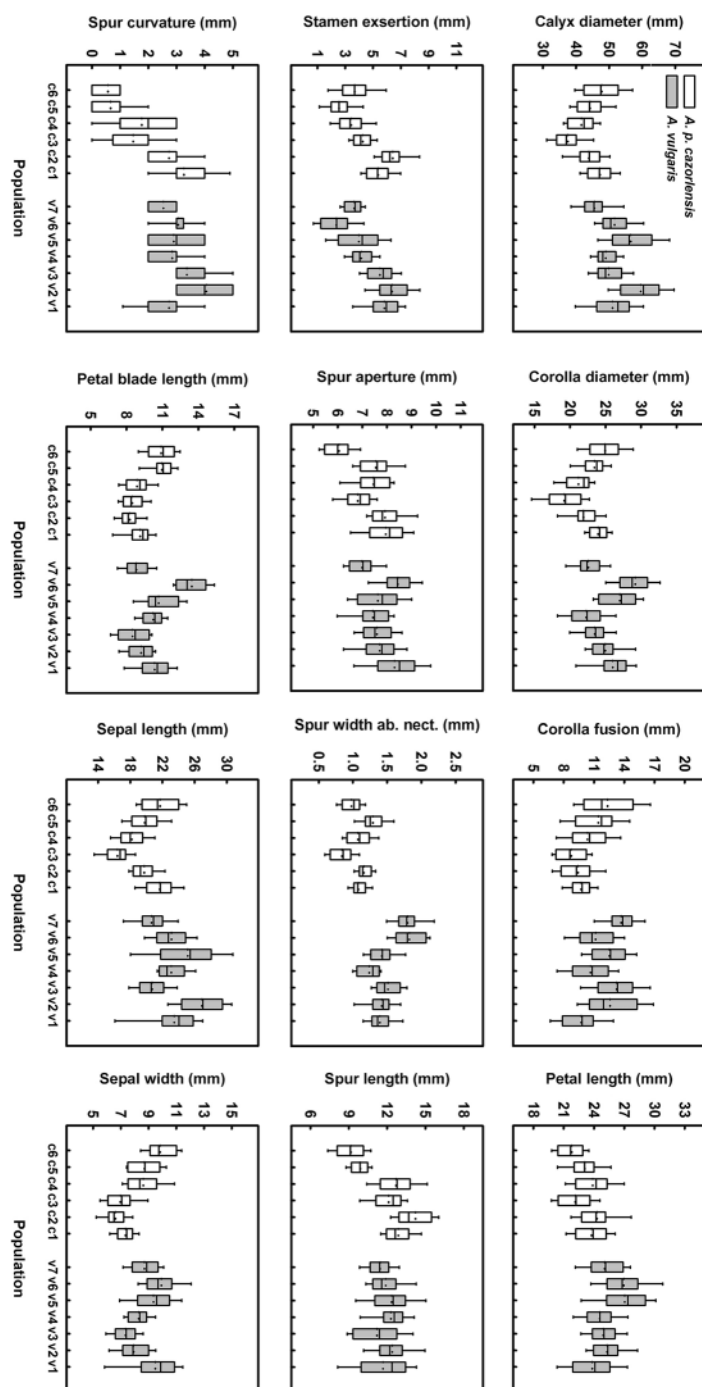
**Variation in floral and vegetative traits among taxa and populations.** At the small spatial scale of this study, we found significant differences between the two taxa, and among the seven *A. vulgaris* and the six *A. p. cazortensis* populations, both for traits considered individually (Figs. 3 and 4) and when all morphological traits measured were treated simultaneously in a multivariate analysis of variance (Table 2). Likewise, when the 12 floral and the ten vegetative traits were analysed separately, significant differences between taxa and among populations within the two taxa were found (Table 2). Mean values of most floral and vegetative traits

overlapped to some extent between populations of both taxa, but *A. vulgaris* plants tend to be larger and have bigger leaves and petals when compared to *A. p. cazortensis* (Figs. 3 and 4).

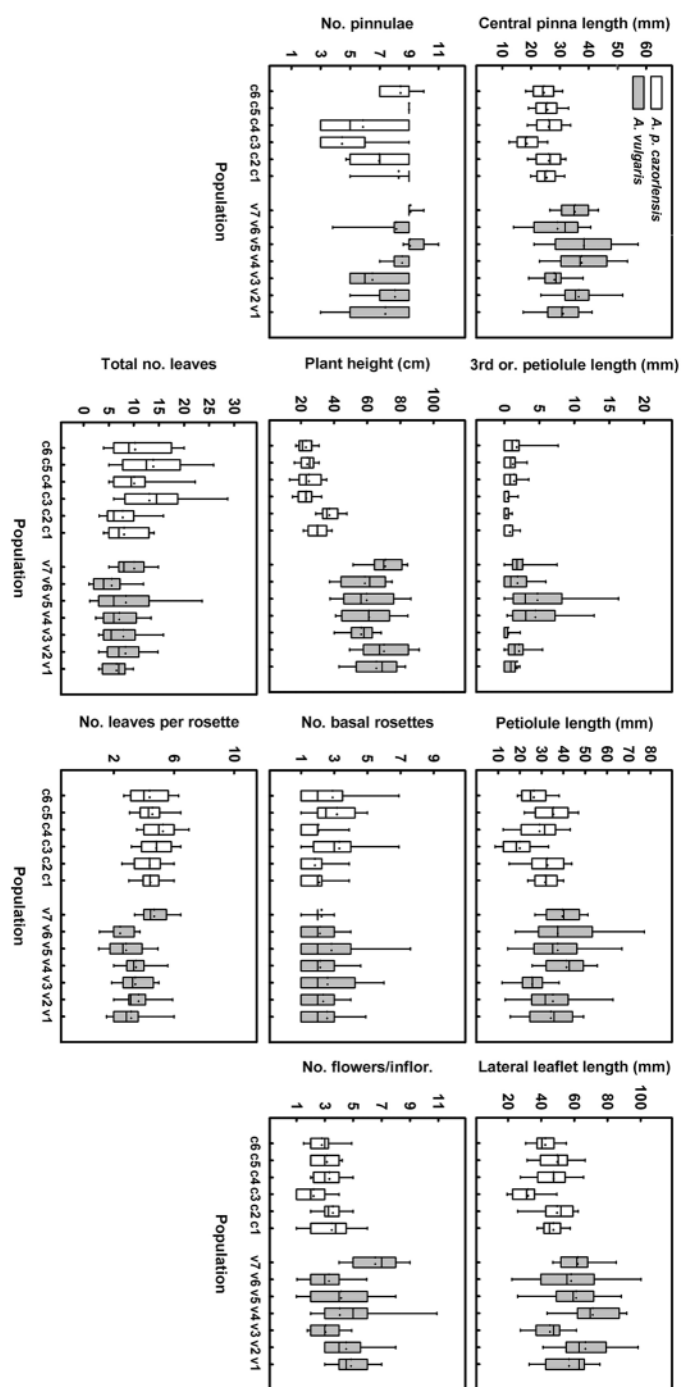
In order to determine which type of traits (floral or vegetative) and which specific characters best discriminated between the two taxa and their populations, canonical discriminant analyses were run for all traits combined and also for each group of traits separately. Figure 5 shows the mean scores on the first two canonical variates with the 75% confidence ellipses for each *Aquilegia* population. When all variables were included, the analysis separated populations into two groups along CV1 (Fig. 5A) corresponding to the two taxa. CV1 accounted for 46% of the between-population variation and together with CV2, explained 68% of the variation. The two variables that contributed the most to CV1 were plant height and length of the petiole (a measure of leaf size). They are plotted in Fig. 6 along with all the other variables, ranked according to their contribution to CV1. Floral traits appear interspersed with vegetative traits and none is particularly important for discrimination between *Aquilegia* taxa and populations (canonical weights of variables are shown in the Appendix).

The lower discriminating power of floral traits is confirmed by comparing the results of separate CDA analyses on floral and vegetative traits. Populations of the two *Aquilegia* taxa appear interspersed, and overlap extensively in the plane defined by floral-based canonical variates (Fig. 5B), while populations of the two species segregate sharply in the plane defined by vegetative-based variates (Fig. 5C). To examine this more closely, we partitioned the variance of scores on CV1 among taxa, among populations within taxa, and within populations, for each of the three CDAs. The variance due to floral differences was mostly due to differences among populations within species (50.3%), and only secondarily between the two taxa (36.2%). In contrast, more than 80% of the variance in





**Fig. 3.** Variation among the six *A. p. cazorlensis* populations (white boxes) and the seven *A. vulgaris* populations (grey boxes) in the 12 floral traits studied. Box plots show the mean (dotted line), median (solid line), 10, 25, 75 and 90% percentiles of the distributions of individual plant measurements. The unusual appearance of the box plots for spur curvature is due to this variable being measured on a semi-quantitative scale (see text). Univariate analyses of variance testing for heterogeneity between taxa and among populations within taxa in trait means are all statistically significant ( $F$ -value  $\geq 279.5$ ,  $P < 0.0001$  in all cases). Refer to Fig. 1 for population locations, and Materials and methods and Fig. 2 for description of traits



**Fig. 4.** Variation among the six *A. p. cazorlensis* populations (white boxes) and the seven *A. vulgaris* populations (grey boxes) in the ten vegetative traits studied. Box plots show the mean (dotted line), median (solid line), 10, 25, 75 and 90% percentiles of the distributions of individual plant measurements. The unusual appearance of the box plots for number of pinnulae and number of basal rosettes is due to these variables being measured on a semi-quantitative scale. Univariate analyses of variance testing for heterogeneity between taxa and among populations within taxa in trait means are all statistically significant ( $F$ -value  $\geq 11.7$ ,  $P < 0.0001$  in all cases). Refer to Fig. 1 for population locations, and Materials and methods and Fig. 2 for description of traits



**Table 2.** Results of multivariate analyses of variance on floral and vegetative data, conducted on all traits combined (“All”) and separately for floral and vegetative traits, testing for differences between *A. vulgaris* and *A. p. cazorlensis* and among populations within the two taxa

Comparison	Type of traits	Wilks $\lambda$	df	<i>P</i>
Between taxa	All	822.9	44, 464	<0.001
	Floral	772.9	24, 558	<0.001
	Vegetative	1037.7	20, 632	<0.001
Among populations				
	<i>A. vulgaris</i>			
	All	24.1	154, 654	<0.001
	Floral	46.0	84, 816	<0.001
	Vegetative	28.4	70, 840	<0.001
<i>A. p. cazorlensis</i>	All	41.2	132, 612	<0.001
	Floral	67.1	72, 685	<0.001
	Vegetative	41.5	60, 806	<0.001

vegetative-based canonical variate scores occurred between the two taxa. The same is true for the CDA that included both types of variables. In all cases, the variance component due to differences among individual plants within populations was comparatively minor.

**Congruency of floral and vegetative trait-variation.** The relative position of each population along CV1 changed little whether only vegetative or only floral traits are used. The population means (centroids) on CV1 in the analysis that included floral variables only (Fig. 5B) are strongly correlated with those in the analysis of the vegetative traits (Fig. 5C), even after controlling for the effect of taxon in a partial correlation analysis (partial  $r_p = -0.699$ ,  $P = 0.0114$ ). The same does not hold true for CV2 (partial  $r_p = -0.473$ ,  $P = 0.1202$ ). This result indicates that the two types of traits vary congruently across populations of both taxa.

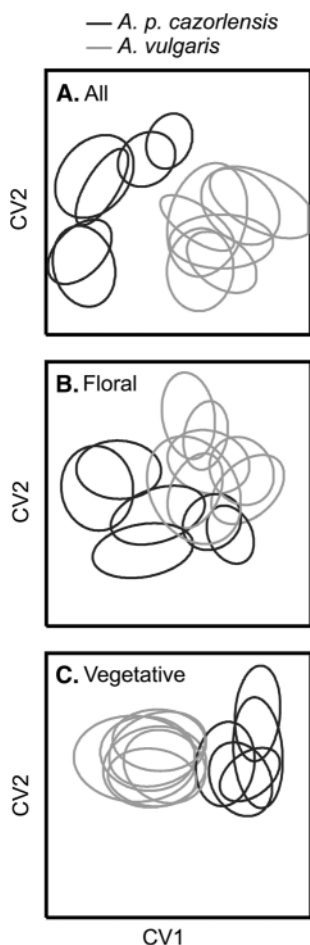
**Geographical patterns.** We found a strong correlation between the population’s position on CV1 (Fig. 5A) and latitude (Fig. 7,  $r_s = 0.775$ ,  $P < 0.005$ ). Separate correlations between the canonical variate scores for floral (Fig. 5B) and vegetative (Fig. 5C) traits and latitude were also significant ( $r_s = 0.747$ ,  $P = 0.005$  and  $r_s = -0.758$ ,  $P < 0.01$ , respectively). The relationship between geographical and morphological distances between populations in the multivariate space was tested by means

of Mantel tests. A geographical distance matrix was constructed based on the UTM coordinates for each population. Morphological distance matrices were constructed with the Euclidean distances between the centroids of the populations (scores on CV1 and CV2) in each of the three CDA. The correlation between the geographical and morphological distance matrices was significant for all the variables combined and for the vegetative variables ( $r = 0.315$ ,  $P = 0.018$ ; and  $r = 0.336$ ,  $P = 0.009$ , respectively), but not for floral variables ( $r = 0.116$ ,  $P = 0.18$ ).

## Discussion

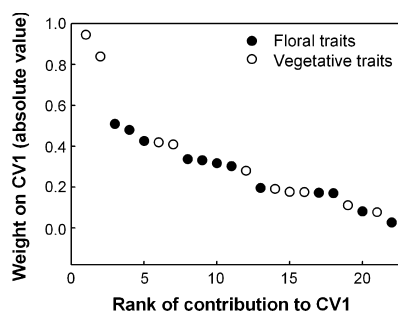
### Interspecific floral vs. vegetative differentiation.

This study has demonstrated that *A. vulgaris* and *A. pyrenaica* subsp. *cazorlensis* are significantly differentiated not only by their morphological floral characters but also by vegetative traits. As we expected, our results indicate that when the relative importance of the two types of traits is compared, only vegetative characters (particularly plant height and petiole length) contribute significantly to the discrimination of the two taxa. Some authors have suggested that in comparison with widespread taxa, narrow endemic plant species are often associated with more stressful habitats (e.g. higher altitudes, relatively sterile

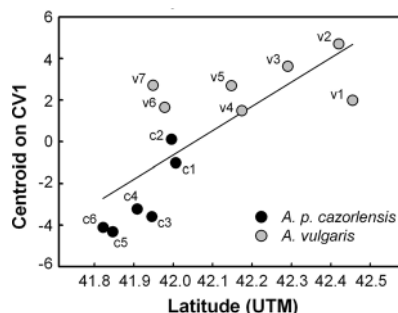


**Fig. 5.** Location of the  $N = 13$  study populations over the plane defined by the first two canonical variates (CV1 and CV2) obtained from Canonical Discriminant Analyses conducted on: **A** all morphometric variables combined, **B** floral variables only, and **C** vegetative variables only. Ellipses are the 75% confidence intervals around the centroid (mean) for each population

soils, or xeric conditions) and exhibit traits reflecting stress-tolerance, such as small stature, high nutrient conservation ability, or low resource acquisition capacity - assessed e.g. by lower specific leaf area, or lower photosynthetic rate - (for a recent review see Lavergne et al. 2004, and references therein). In our study, plants of the narrow endemic *A. p. cazorlensis* show a clear pattern of ecological differentiation from its widespread congener *A. vulgaris*. Furthermore, *A. p. cazorlensis* grows in more stressful conditions (i.e. shallow



**Fig. 6.** Correlations of floral and vegetative traits with the first canonical variate (CV1) in Fig. 3A. Points represent each of the 22 variables (filled if floral, empty for vegetative) ranked in decreasing order of correlation with CV1



**Fig. 7.** Relation between latitude and mean scores of *Aquilegia* populations on the first canonical variate (CV1) resulting from a canonical discriminant analysis on floral and vegetative variables combined (Fig. 3A). The line is the least-squares fitted regression

sandy soils at rifts of limestone outcrops and cliff bases, in sites with a high bedrock and block cover and fewer coexisting species) than *A. vulgaris*. The smaller plant size of *A. p. cazorlensis* found in this study could indicate that this species may have lower competitive ability for space and light than its widespread congener, as has been shown for *A. viscosa* and *A. vulgaris* along with other 19 pairs of endemic and widespread plant species from western Mediterranean flora (Lavergne et al. 2003, 2004). In addition, if *A. p. cazorlensis* had a stress tolerator species strategy, a lower value of specific leaf area than its widespread congener is predicted (Westoby 1998).

Although data on specific leaf area for both taxa are required to confirm this hypothesis, the finding that leaves of *A. p. cazorlensis* tend to be smaller than those of *A. vulgaris* could be suggestive of a stress tolerator strategy.

Even though more detailed quantitative assessments of the pollinator fauna of both taxa are required (but see also Lavergne et al. 2005), our preliminary data on floral visitors in *A. vulgaris* and *A. p. cazorlensis* indicate that the two taxa are pollinated by essentially the same species of bumblebees, and that other occasional visitors like small bees or syrphid flies are also shared by both species. The weakly specialized spectrum of pollinators shared by *A. vulgaris* and *A. p. cazorlensis*, along with the similarity in floral morphology of the two species, would suggest that differentiation has probably occurred in this instance without divergence in pollinator specialization.

Although our results are preliminary and need to be confirmed with more detailed investigations, the fact that morphological differentiation between the two studied taxa are only due to vegetative -and not to floral- characters seems to be inconsistent with the current views of the radiation process in the genus *Aquilegia*. The remarkable and wide variation in floral morphology within this genus has led to the suggestion that diversification in floral characters has been central to the differentiation within the genus (Grant 1952, Hodges and Arnold 1994a), although the role of divergence in habitat requirements cannot be completely ruled out (Chase and Raven 1975, Hodges and Arnold 1994b). It has been also proposed that the extremely rapid radiation of the genus in North America was triggered by the evolution of nectar spurs (Hodges and Arnold 1994a, 1995; Hodges 1997), which facilitated the specialization on different pollinators. However, it is important to note that these hypotheses are supported basically by results from studies performed on North American *Aquilegia* taxa, where clades with broadly divergent floral characters coexist (Grant

1952). For instance, while no vegetative features consistently separated North American *A. formosa* and *A. pubescens* (Chase and Raven 1975), which are pollinated respectively by hummingbirds and hawkmoths, sets of nonoverlapping floral characters undoubtedly differentiated them (Grant 1952, Hodges and Arnold 1994b, Hodges et al. 2002). Floral differentiation has been also observed between *A. elegantula* and *A. caerulea* in the mountains of Colorado (Miller 1978), although no mention to vegetative traits was done in this study. Although further investigations on other Eurasian *Aquilegia* taxa are needed to confirm our results, we suggest that selective pressures imposed by pollinators may have been less decisive in the radiation of the genus *Aquilegia* in Europe than in North America. This hypothesis is supported by our finding that no nectar spur dimension was particularly important in differentiating between species, which also implicates that spurs might not have been a key aspect of the radiation of European *Aquilegia*.

**Intraspecific variation.** Significant differences in floral and vegetative morphological features also occur among populations within both taxa, and the two types of traits vary congruently across populations of a given taxon. In other words, populations with smaller plants and leaves are more likely to have also undersized flowers (and viceversa) both in *A. vulgaris* and *A. p. cazorlensis* (see Figs. 3 and 4). Furthermore, this result indicates that in both taxa vegetative characters are somewhat allometrically or pleiotropically linked to floral traits, a pattern that has been previously found in many other plant species (Bond and Midgley 1988, Midgley and Bond 1989, Conner and Sterling 1996, Hornung-Leoni and Sosa 2006).

Although morphological differentiation between *A. vulgaris* and *A. p. cazorlensis* is mainly due to vegetative traits, differences among populations within taxa are mostly explained by variation in floral traits. Therefore, morphological divergence between the

two taxa cannot be interpreted as a scaled-up extension of among-population differences occurring within taxa, i.e., it appears that patterns of micro- and macro-evolutionary differentiation do not concur, at least in the “snapshot” of current conditions represented by our study. Two mutually non-exclusive processes may account for the observed inter-population differences in floral characters: adaptive local differentiation due to divergent selection, and/or neutral phenotypic variation related to genetic drift. Natural selection may favour different floral phenotypes in each population in response to differences in selective regimes among localities (Wright 1931). Alternatively, if populations were founded by a few individuals, or if the populations were small or spatially isolated, differentiation could reflect neutral phenotypic variation due to genetic drift (Dobzhansky 1970; Slatkin 1987, 1993). In the present instance, some indirect evidence suggests that while population differentiation in floral characters could mainly be a consequence of genetic drift, divergence in vegetative characters could be mainly due to natural selection, as discussed below.

**Geographical patterns.** Considering all the traits studied simultaneously, we found not only a multivariate differentiation between *A. vulgaris* and *A. p. cazorlensis* and among populations within taxa, but also that they follow a clinal latitudinal pattern along the Cazorla-Segura-Las Villas mountains, which would suggest adaptation at a local scale to changing environmental conditions. Multivariate vegetative, but not floral, similarity between populations could be predicted from geographical distance, indicating that populations that are geographically closer tend to be more similar in vegetative traits, but not in their floral characteristics, than those that are more distant. This pattern of distance-independent floral differentiation among populations is similar to the “random patchwork” pattern of floral morphology described by Herrera (1990) for *Viola cazorlensis*, another narrowly endemic species with isolated populations distributed over the study region.

Because nearby populations are expected to share similar environmental conditions, and similar adaptations are more likely to evolve as a response to similar selective pressures, the fact that in *A. vulgaris* and *A. p. cazorlensis* closer populations were more similar in their vegetative traits than more distant populations could be the result of the action of natural selection for a local vegetative optimum, although phenotypic plasticity could not be completely ruled out. This contrasts with the situation for floral characteristics, as it seems that floral differentiation among populations is independent of local conditions and do not conform to a latitudinal gradient. These findings suggest that population differentiation in floral characters could be a consequence of random genetic drift occurring in relatively small or isolated populations, and in conditions of restricted gene flow. This hypothesis is supported by various lines of evidence. In the Cazorla-Segura-Las Villas mountains, local populations of *A. vulgaris* and *A. p. cazorlensis* are fairly isolated by both topographical (because of the rugged topography of the region) and ecological barriers, since their favorable, humid microhabitats occur as sparse, small islands amidst extensive expanses of dry Mediterranean vegetation. This distribution is similar to that reported by Strand et al. (1996) for other species of *Aquilegia* in the arid southwestern United States and México. In these conditions, gene flow among populations via seeds and/or pollen is expected to be low, both because plants lack any specialized mechanism of long distance seed dispersal, and because flights among populations of their main pollinators (*Bombus* species) will probably be infrequent (Chapman et al. 2003 and references therein). Preliminary results of genetic analyses on several of the populations included in this study using microsatellite markers reveal extreme genetic differentiation and restricted gene flow between nearby populations (M. C. Castellanos, unpubl. data), which is consistent with the hypothesis of floral differentiation by genetic drift. Likewise, using chloroplast DNA

markers, Strand et al. (1996) found restricted gene flow and high degree of genetic differentiation for populations of *Aquilegia longissima* and *A. chrysantha* in southwestern United States and northern México with a similar island-like population structure. In this study, genetic relatedness among populations seems attributable to recent historical relationships rather than to ongoing gene flow.

In conclusion, the study of morphological differentiation among populations of *A. vulgaris* and *A. p. cazorlensis* along the geographical region where both taxa enter in contact has revealed that multivariate patterns of floral and vegetative differentiation between taxa and among populations within taxa are not concordant, which suggests that abiotic factors may have been more important than pollination-related ones in promoting diversification. These results suggest that, to understand the diversification of European

*Aquilegia*, investigations on the nature of evolutionary forces and ecological conditions involved in the differentiation of vegetative traits may prove equally or more important than studies on diversification of pollination-related features.

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**Appendix.** Weights on the first canonical variates of the 22 traits included in the analysis, standardized by within variances. Most important traits in each canonical variate are in bold

Trait type	Trait	All		Floral		Vegetative	
		CV1	CV2	CV1	CV2	CV1	CV2
Floral	Calyx diameter	-0.170	0.084	-0.038	-0.193		
	Corolla diameter	0.026	-0.189	0.059	0.284		
	Corolla "fusion"	0.172	-0.043	0.066	0.018		
	Petal length	0.508	0.122	<b>0.638</b>	0.381		
	Stamen exertion	0.316	0.463	0.457	-0.29		
	Spur aperture	-0.302	0.305	-0.053	-0.32		
	Spur width above nectary	0.336	-0.357	0.271	<b>0.776</b>		
	Spur length	-0.081	<b>0.525</b>	0.1	-0.501		
	Spur curvature	0.479	0.399	<b>0.707</b>	0.059		
	Petal blade length	-0.195	-0.110	-0.277	0.027		
	Sepal length	0.331	-0.085	0.117	0.102		
	Sepal width	-0.425	-0.423	-0.633	0.094		
	Vegetative	Length of petiolule	<b>-0.838</b>	0.142			<b>0.892</b>
Length of central pinna		0.191	-0.263			-0.441	-0.561
Length of 3rd order petiole		-0.177	-0.075			0.212	<b>0.594</b>
Length of lateral leaflet		0.280	0.260			-0.118	-0.107
No. of pinnulae		0.111	-0.220			-0.247	<b>-0.958</b>
Plant height		<b>0.945</b>	<b>-0.480</b>			<b>-1.295</b>	0.474
No. of basal rosettes		0.175	0.095			0.073	0.046
No. of flowers/inflorescence		-0.408	0.183			0.443	-0.57
Total no. of leaves		-0.418	-0.130			0.189	0.167
No. leaves per rosette		0.077	0.205			0.166	0.054

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