Intra-population comparison of vegetative and floral trait heritabilities estimated from molecular markers in wild *Aquilegia* populations

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Abstract

Measuring heritable genetic variation is important for understanding patterns of trait evolution in wild populations, and yet studies of quantitative genetic parameters estimated directly in the field are limited by logistic constraints, such as the difficulties of inferring relatedness among individuals in the wild. Marker-based approaches have received attention because they can potentially be applied directly to wild populations. For long-lived, self-compatible plant species where pedigrees are inadequate, the regression-based method proposed by Ritland has the appeal of estimating heritabilities from marker-based estimates of relatedness. The method has been difficult to implement in some plant populations, however, because it requires significant variance in relatedness across the population. Here we show that the method can be readily applied to compare the ability of different traits to respond to selection, within populations. For several taxa of the perennial herb genus *Aquilegia*, we estimated heritabilities of floral and vegetative traits and, combined with estimates of natural selection, compared the ability to respond to selection of both types of traits under current conditions. The intra-population comparisons showed that vegetative traits have a higher potential for evolution, because although they are as heritable as floral traits, selection on them is stronger. These patterns of potential evolution are consistent with macroevolutionary trends in the European lineage of the genus.

Introduction

The presence of genetic variation is a precondition for the evolution of any trait, but in spite of its importance in predicting a character's ability to respond to selection, measuring heritability and other quantitative genetic parameters in wild plant populations under natural conditions is still rarely done. This is mostly the consequence of the inherent difficulty in assigning genealogical relationships among wild individuals. Traditionally, plant quantitative genetic studies are performed with individuals of known pedigree grown under controlled conditions or transplanted to the field (Riska *et al.* 1989; Shaw 1986). These studies are the basis of what we know today of trait inheritance and genetic correlations, and yet they do not necessarily reflect how traits are expressed in natural conditions (Campbell 1996; Conner *et al.* 2003; Winn 2004). Marker-based methods that can be applied directly to wild populations to estimate relationship and quantitative genetic parameters have therefore received much attention recently, particularly for animal populations, and to a smaller extent, to plants as well (Ritland 2000; Garant & Kruuk 2005).

Marker-based field measurements of heritability (h^2) and other quantitative genetic parameters have several advantages over controlled experimental studies. The first is that they incorporate the effects of environmental variation and natural mating patterns on the phenotypes that actually face natural selection. Studies under controlled conditions have been shown to underestimate environmental effects, and therefore to inflate h^2 values compared to those estimated in the field (Montalvo & Shaw 1994; Schoen *et al.* 1994; Conner *et al.* 2003; but see Ritland & Ritland 1996). Second, marker-based methods can be the only alternative for the studying the vast number of long-lived plant species where controlled experiments are impractical or unfeasible, particularly if individuals are large or take years to reproduce (Ritland & Travis 2004;

61 Andrew *et al.* 2005). In addition, controlled conditions are also inappropriate for plants
62 with mixed mating systems that are difficult to emulate with manual pollinations. A
63 third advantage of field methods is that they can be applied in studies that require large
64 spatial or temporal scales that cannot be addressed with controlled crosses, such as to
65 detect whether trait heritabilities change across years in a population, or to estimate
66 genetic parameters in multiple populations as required by studies of character
67 divergence. It is therefore important to test marker-based methods and their
68 applicability to different plant populations.
69 Analytical tools have been developed for inference of quantitative genetic parameters
70 for field data sets both with partial or complete pedigrees, and for the estimation of
71 relatedness with no previous knowledge or assumptions about a population's
72 genealogical structure (reviewed by Garant & Kruuk 2005). In principle, the
73 reconstruction of a pedigree is best for the estimation of quantitative genetic parameters
74 in a natural population (e.g. the "animal model"; Thomas *et al.* 2002; Garant & Kruuk
75 2005). Pedigree reconstruction methods require at least some previous information,
76 such as discriminating adults from offspring, or behavioural observations of possible
77 matings. Even with such information, marker-based pedigree reconstruction methods
78 for wild populations are not perfect (Butler *et al.* 2004), as they are sensitive to data
79 errors and mutations. Furthermore, under some circumstances pedigrees might not even
80 be desirable. For many long-lived plant species where individuals can self-pollinate and
81 generations are indistinguishable in the field, the reconstruction of a pedigree is not only
82 troublesome, but it is likely that a continuous measure of relatedness is even a better
83 representation of relationships.
84 For such plant populations, one pedigree-free alternative for estimating heritabilities
85 is Ritland (2006)'s regression-based method, which relies on pairwise relatedness

86 estimates and phenotypic similarity to estimate heritability of the traits of interest
87 through linear regression. Several marker-based estimators of relatedness have been
88 developed (reviewed by Blouin 2003), and although they are not error-free, they can be
89 used in Ritland's regression approach in combination with an estimation of the actual
90 variance in relatedness (Var(r)) in the population. The method requires that populations
91 harbour significant Var(r) (*i.e* that there is a mixture pairs of individuals related to
92 various degrees), which is one of the major obstacles for its application to wild
93 populations (Ritland 1996; Csilléry *et al.* 2006; Shikano 2008).
94 In tests involving populations of obligate outbreeders where authors have compared
95 h^2 values estimated by Ritland's method and other pedigree-based methods, Ritland's
96 estimates are often found to be inaccurate (Thomas *et al.* 2002; Coltman 2005; Frentiu
97 *et al.* 2008; Van Horn *et al.* 2008; but see DiBattista *et al.* 2008). However, outbred
98 vertebrates often present low variance in relatedness (Csilléry *et al.* 2006). In other
99 organisms, evidence that Ritland-based estimations can be reasonably accurate is slowly
100 accumulating (Andrew *et al.* 2005; DiBattista *et al.* 2008; Herrera & Bazaga 2009;
101 Anderson *et al.* 2010). Andrew *et al.* (2005), for instance, successfully estimated
102 heritabilities of defence chemicals in a population of a long-lived tree species and
103 argued that, provided that there is enough variance in relatedness, it can be a useful
104 approach. Yet even if this requirement is met, estimation of heritabilities using
105 molecular markers can suffer from low statistical power (Ritland 1996; Rodríguez-
106 Ramilo *et al.* 2007), as is the case for the estimation of quantitative parameters with
107 other methods (Mitchell-Olds & Rutledge 1986). Large sample sizes and highly
108 polymorphic markers might be required, and this might have helped prevent the use of
109 this method more extensively. However, small population sizes is the rule for many
110 plant species. Here, we test the regression approach for small wild populations, aiming

111 to make intrapopulation comparisons of trait heritabilities, rather than trying to compare
112 estimates across-taxa. Even if estimates are not accurate, and even outside of the
113 theoretical range, we show that this approach can be useful when within-population
114 comparisons are needed.

115 We estimated heritabilities of floral and vegetative traits in Iberian populations of the
116 herb genus *Aquilegia* (Ranunculaceae) in order to compare the ability to respond to
117 selection of both types of traits within each population. In *Aquilegia*, two contrasting,
118 recent radiations have occurred simultaneously in North America and Europe, after the
119 colonization by an Asian ancestor, and both have given rise to about the same number
120 of species (Bastida et al. 2010). In the New World, the diversification of the group has
121 been associated with floral adaptation to different pollinators (Grant 1952; Hodges &
122 Arnold 1995). Specialized floral morphological features, mainly the elongated petals
123 that form a nectariferous spur, help determine the identity of the pollinators that can
124 reach the nectar, and lead to rapid floral specialization on bees, hummingbirds or moths,
125 and ultimately to reproductive isolation (Whittall & Hodges 2007). Even though habitat
126 characteristics also differ between North American *Aquilegia* species (Chase & Raven
127 1975; Hodges & Arnold 1994), the major role of pollinators is evident because shifts to
128 different pollinators are common in the phylogeny (Whittall & Hodges 2007) and recent
129 speciation events may have occurred in sympatry (Bastida et al. 2010). In contrast,
130 pollinator shifts are absent from the European lineage (all species are pollinated by
131 bumblebees and other bees), while habitat shifts have been common (Bastida et al.
132 2010). In addition, at least in some groups, vegetative traits are more important than
133 floral traits in differentiating species (Medrano et al. 2006). A recent study further
134 shows that vegetative traits in Iberian columbines have diverged in response to
135 adaptation to different habitats (Alcántara et al. 2010). Still, floral characteristics do

vary among species and even among populations of the same species (Gafta et al. 2006; Medrano et al. 2006).

In this study we compare the current ability to respond to selection of floral versus vegetative traits in European *Aquilegia* populations in two widely distributed subspecies (*Aquilegia vulgaris vulgaris* and *A. pyrenaica pyrenaica*) and their endemic sister taxa (*A. v. nevadensis* and *A. p. cazorlensis*). We test Ritland's methods in these small, but substructured populations. Although current response to selection does not necessarily directly inform us about macroevolutionary patterns, we also aimed to find out if current microevolutionary patterns in populations of different taxa agree with the macroevolutionary patterns in the European lineage of the genus.

Materials and methods

Study species

Columbines are perennial rhizomatous herbs with one or a few basal rosettes that can bear erect, paniculate inflorescences with one to several flowers. This study included 15 *Aquilegia* populations belonging to two subspecies of each of the two most common species in the Iberian Peninsula, *A. vulgaris* and *A. pyrenaica* (Table 1). *Aquilegia vulgaris* is widely distributed throughout Eurasian mountain forests, open woodlands and meadows. *A. vulgaris* subsp. *vulgaris* is the most common subspecies; populations in this study grow along stream margins or poorly drained open meadows around springs from 1100 to 1700 m of elevation, but this subspecies can be found at lower elevations including sea level. In contrast, A. *vulgaris* subsp. *nevadensis* is restricted to the Sierras Béticas of Southern Spain, where populations grow on moist forest soils but also in wet alpine meadows and scrublands, between 1500 and 2100 m of altitude. *Aquilegia pyrenaica* subsp. *pyrenaica* has a wide distribution through the Pyrenees and

Cantabrian Mountains in Northern Spain, with high altitude populations growing in alpine meadows, rocky outcrops and rocky grasslands between 1200 and 2250 m of altitude. Its congeneric A. *pyrenaica* subsp. *cazorlensis* is in contrast a narrow endemic to the Sierras of Cazorla and El Pozo, in Southeastern Spain. The few known populations grow between 1400 and 2000 m of altitude in rifts of limestone outcrops and on sandy soils in shady, damp sites at cliff bases. Details on the populations in this study can be found in Table 1.

An average of 45 (21-60) mature individuals in each population were selected for this study in the blooming season of 2007. Sample sizes were constrained by the sizes of the populations, which are very small in some cases (Table 1), as well as the number of blooming individuals. From each individual we collected fresh leaves, a single petal and sepal, and a ripe fruit capsule. The leaves were silica gel-dried for DNA extraction.

Phenotypic characterization and seed production

Digital images of dried petals and sepals were used to measure six floral traits for each individual plant. *Aquilegia* sepals tend to be large and colourful, and function as advertisement along with the petals. The petals present elongated spurs that form a tube and get narrower towards the nectariferous tip. We measured three traits potentially related to floral advertisement: sepal width, sepal length, and petal blade length; and three traits related to the mechanical interaction of the flower with the floral visitor: spur length, spur width at its aperture and spur width above the nectary. Measurements were taken on calibrated digital images using SigmaScan Pro (version 5.0). Additionally, six vegetative traits, measured for the same individuals by Alcántara et al. (2010) are also used here for estimations of heritability in comparison with floral traits: height of the tallest inflorescence, total number of leaves, length of the longest leaf, number of

187 flowers per inflorescence, specific leaf area, and density of non-glandular pubescence in
188 leaves. Specific leaf area was determined in the laboratory from a sample of the longest
189 leaf, and the density of pubescence in the leaves was estimated under dissection
190 binoculars from fresh epidermal tissue (details in Alcántara et al. 2010). In addition,
191 seed production of each individual plant was estimated as the product of a) the number
192 of healthy seeds produced in a single fruit collected in the field and b) the total number
193 of ripe carpels produced by the plant.
194
195 *DNA extraction and microsatellite analysis*
196 DNA of each individual plant was extracted from dried leaf material using the
197 Speedtools Plant DNA Extraction Kit (Biotools, Madrid, Spain). We amplified 10
198 microsatellite loci, chosen among those developed for North American *Aquilegia* by
199 (Yang *et al.* 2005), with some modifications on the PCR protocol. For 20 μL PCRs, we
200 added template DNA, 0.25 μM of each primer (forward primers were labelled with
201 flourophores), 0.1mM of each dNTP, 1 unit of Taq polymerase, 3.5 mM of $MgCl_2$, and
202 1x reaction buffer. PCR reactions started with a 4-min denaturation phase at 94 ºC,
203 flowed by 38 cycles of 94 ºC, 45 s; 56-62 ºC, 45 s; 72 ºC, 45 s, and a final extension
204 step of 72 ºC for 10 minutes. Fragment analysis was carried out on an ABI 3730 DNA
205 Analyzer (Applied Biosystems). We visualized peaks with Genemapper Software v.4.0
206 (Applied Biosystems, Foster City, CA) and used MstatAlelle in R (Alberto 2009)
207 combined with manual checking for allele scoring. One locus (10-15) failed to amplify
208 in two *Aquilegia p. cazorlensis* populations (Table S1, Supporting information).
209 For each genotyped population, we checked for linkage disequilibrium (LD) and
210 deviations from Hardy-Weinberg equilibrium in Genepop 4.0 (Rousset 2008). There
211 was no evidence of LD, as no pair of loci showed a consistent correlation across

populations within each species. Homozygote excess compared to HW expectations suggested potential null alleles for several loci in some populations. In most cases, several loci showed deviations within a population. Except for one locus (50-21) in one population (Cabañas), however, there was no evidence of homozygotes for the potential null alleles, as calculated from their frequencies estimated in ML-RELATE (Kalinowski et al. 2006). Because populations show deviations from equilibrium in several loci, a more likely explanation is population substructure, as expected if the populations are divided into a series of closely related or inbred family groups. This is likely the case for our small, poorly-dispersed, self-compatible columbines. Population substructure is actually useful for the relatedness estimations intended in this work (see below). Exclusion of the locus with evidence of null alleles in Cabañas had no qualitative effect on the analyses.

Estimates of relatedness

Molecular marker data allow for the estimation of relatedness among individuals in a population, provided enough polymorphism exists. Several estimators of relatedness have been developed and their effectiveness depends on the populations of study (Van de Casteele *et al.* 2001; Blouin 2003). The later authors suggested using simulations based on the allele frequencies of the study populations to decide on the best estimator for relatedness. We used Monte-Carlo simulations implemented in the software Mark (Ritland 1996) to determine the best estimator of relatedness for each one of our *Aquilegia* populations. Ritland's R estimator provided the most reliable estimation (lowest error) of relatedness (r) and actual variance of relatedness (Var(r)) for all populations, when compared to Queller and Goodnight (1989), Lynch and Ritland

(1999), and Wang (2002) methods. We estimated relatedness *r* for all pairs of individuals of each population using Mark, as well as the population's Var(*r*).

Estimates of heritability

The relatedness values inferred from microsatellite markers can next be correlated with phenotypic similarity to estimate heritability of individual traits. We used Ritland's regression method to estimate heritabilities, as implemented in the program Mark (v. 3.1). Ritland's (1996) method relies on pairwise relatedness estimates and pairwise phenotypic similarity to estimate heritability of the traits of interest through linear regression. Pairwise similarity for individuals *i* and *j* and trait Y is calculated as

$$Z_{ij} = (Y_i - U)(Y_j - U)/V$$

where *U* and *V* are the mean and variance of the phenotypic trait in the sample. The average Z_{ij} among all pairs is the phenotypic correlation and can be estimated as a combination of shared alleles and environments:

$$Z_{ij} = 2r_{ij}\, h^2 + r_e + e_{ij}$$

where r_{ij} is the relatedness, r_e is a correlation due to shared environment, and e_{ij} is the error. Incorporating a correlation due to shared environments is important, because in natural populations relatives might be clustered in space and phenotypic variance caused by environmental factors could confound the phenotypic correlation between them. Over all pairs of individuals in the sample, the estimated heritability is then

$h^2 = \text{cov}(Z_{ij}, r_{ij})/2\ Var(r_{ij})$

where $Var(r_{ij})$ is the actual variance in relatedness, a population parameter that needs to be high for this method to work. $Var(r_{ij})$ is estimated with a weighted ANOVA of estimates of relatedness of independent loci (see details in Ritland 1996). The significance of all estimates was calculated with the percentile method, based on 1000 bootstrap replications where individuals are resampled. An estimate was considered significantly different from zero if the 95% confidence interval was higher than zero.

We compared values of estimated h^2 for floral and vegetative traits within populations. The accuracy of estimates of h^2 decreases as the square of $Var(r_{ij})$. Since our estimates of actual variance in relatedness span one order of magnitude among populations (from 0.002 to 0.03; see results), the differences in accuracy span two orders of magnitude. This strongly prevents against comparisons of the estimated values of heritability among populations or taxa. However, this would not affect comparisons of heritability estimates for different traits within populations as long as $Var(r_{ij})$ is kept constant within population (i.e. the same set of individuals is used to estimate h^2 in all traits). On the other hand, we did not attempt to calculate genetic correlations among traits for our populations, as the error of Ritland's method for this parameter is even larger than that of heritability. Instead, we rely on phenotypic correlations as an indication of potential correlated evolution of floral and vegetative traits.

Estimates of selection

We estimated directional selection in each *Aquilegia* population for the study year, using total seed production as a proxy for female reproductive success. Our sample sizes per population were insufficient for a joint analysis of directional selection on 12

traits; alternatively, we used principal components analysis (PCA) to generate new, uncorrelated floral and vegetative variables. We ran PCAs separately on the six floral and the six vegetative traits, log- or square root-transformed as necessary. The resulting two main principal components (PCs) of the floral ordination account together for 78% of the across-population variance, while the two main vegetative factors account for 69% of the variance. In both cases, all traits correlate strongly with the first or the second factor of its respective ordination (Table S2, Supporting information). Discarded PCs had eigenvalues <1 (Kaiser-Guttman criterion for exclusion; Jackson 1993).

We then used these four PCs as composite variables to obtain estimates of phenotypic selection using the approach by Lande and Arnold (1983). PCs were standardized to zero mean and unit variance and used to estimate directional selection gradients (β) for each *Aquilegia* population using multiple regression analysis on relative seed production. These gradients measure the strength of direct selection on each PC independent from the others. Individual plants with missing trait values were excluded from the PCAs, and two populations with low resulting sample sizes were excluded altogether (B. Jabalises and Garrotegordo). To simplify our assessment of ability to respond to selection of the PC variables, we calculated heritabilities of PCs as well, using the scores of each PC as individual values. Estimating genetic parameters on PC factors is common practice on sets of traits that are potentially genetically correlated (see e.g. Keller et al. 2001).

Results

Phenotypic correlations

In total, we characterized 689 individuals from 15 *Aquilegia* populations belonging to different subspecies of the most common Iberian species, A. *vulgaris* and *A. pyrenaica* (Table 1; see Table S3, Supporting information for mean values). All

312 subspecies and also all populations within subspecies differ from each other in all six
313 floral traits measured (Table 2), as they do for six vegetative traits (Alcántara et al.
314 2010).
315 We estimated phenotypic correlations among ln-transformed traits for each
316 population. The actual pairs of traits that are significantly correlated vary from one
317 population to another, but two general patterns hold for all 15 populations (see Table
318 S4, Supporting information, for the correlation matrix of one *A. v. vulgaris* population,
319 Jabalises, as an example): floral traits are more likely to be correlated among themselves
320 than vegetative traits, and floral and vegetative traits are only occasionally correlated.
321 For all populations, on average, 10 (± 2.9) of the 12 possible correlations among floral
322 traits are significant (and positive), while 4.2 (± 1.5) out of 12 of vegetative traits are.
323 Finally, 7.6 (± 5.1) of the 36 possible correlations between floral and vegetative traits
324 are significant and can be either positive or negative.
325
326 *Relatedness*
327 Two *A. v. vulgaris* populations (Garrotegordo and F. Reina) with very low allelic
328 variation were excluded from the molecular analyses, because error estimation of
329 relatedness increases highly in such cases. In general, we found high values of mean
330 pairwise relatedness, *r,* for the remaining 13 populations, which varied from 0.137 and
331 0.388 and were uncorrelated with population size (Fig. 1; Pearson r_p=-0.14, p= 0.65).
332 Relatedness was particularly high for A. v. *nevadensis* populations. The variance in
333 relatedness, Var(*r*), was significantly greater than zero in all populations and ranged
334 between 0.002-0.03.
335
336 *Heritability*

337 The significant values of Var(r) allowed us to test for statistical significance of
338 heritability estimates for all traits in 13 populations. Most traits in most populations
339 showed h^2 values that were not significantly different from zero, with some exceptions
340 (Table 3). Estimates of h^2 outside of the theoretical range ($0<h^2<1$) are a consequence of
341 Ritland's method and would represent a problem if we were interested in accurate h^2
342 values, but in this case we focus on intra-population comparisons. Due to space
343 limitations, table 3 does not include the values for r_e, the correlation due to shared
344 environment included in the model. This correlation was not significantly higher than
345 zero in any of the traits or populations, indicating that there were no local environmental
346 effects (i.e. patchiness) that differentially affected related individuals growing close to
347 each other.
348 In general, vegetative and floral traits show similar h^2 values: in a comparison of six
349 floral versus six vegetative traits for all populations using paired t-tests (which restrict
350 comparisons to within-population), only 1 of the 36 contrasts was significant (sepal
351 length versus SLA). When comparing mean values of floral versus vegetative traits
352 within each population we found that in only two populations, each of a different
353 species, floral traits were more heritable when compared to vegetative traits of the same
354 population (t=2.306, p=0.028 for B. Canal and t=3.27, p=0.008 for Cortijuela). When
355 focusing on the significance of heritability values only, a notable trend is that for the
356 two widespread taxa, *A. v. vulgaris* and *A.p. pyrenaica*, there is essentially no trait with
357 detectable heritability in any population. All but one of the heritable traits we could
358 detect are in the more restricted, endemic subspecies.
359
360
361

362 *Phenotypic selection and ability to respond to selection*

363 Condensing the variation in floral and vegetative traits in separate composite PC
364 variables seems appropriate, because floral traits are highly correlated among each other
365 and only occasionally with vegetative traits. Directional selection gradients on PC
366 factors were more often significant for vegetative than for floral traits. Out of the 26
367 gradients in each analysis (13 populations and 2 factors), only one was significant for
368 floral factors, while 13 were significant for vegetative factors (Table 4). In other words,
369 during the study year, floral traits were under selection only in Cabañas, while
370 vegetative traits showed directional selection in nine populations, including Cabañas.
371 Moreover, the mean strength of selection, estimated as the mean absolute value of
372 selection gradients across populations, was higher for the two vegetative factors (0.32
373 and 0.32) than for the floral factors (0.20 and 0.09).

374 Analysis of heritability in floral and vegetative PCs showed similar patterns as for
375 individual traits (Table 5; compare to Table 3). Within-population paired comparisons
376 between floral and vegetative heritability values found no significant differences, and
377 only a few of the h^2 estimates are significantly different from zero. Table 5 includes also
378 the values of r_e, the average correlation between individuals caused by shared
379 environment. Again, this correlation was not significant for any population. The
380 potential for evolution of current traits is a function of the heritability values and the
381 strength of natural selection on each trait. A qualitative assessment of both h^2 estimates
382 and selection gradients suggest that vegetative traits had higher potential for evolution
383 than floral traits in the same populations under the current selection regimes, even if
384 both types of traits are heritable, because selection is weaker and infrequent on floral
385 characteristics.

386

Discussion

The inherent logistic difficulties of direct field estimations of trait heritability have prevented their extended use in plant evolutionary studies. In this study we were able to estimate heritabilities of floral and vegetative traits directly in 13 *Aquilegia* populations without any previous information on population structure. Although our estimated h^2 values are not directly comparable to other species or between populations, the within-population approach that we intended showed that there is no overall difference in heritabilities between floral and vegetative traits. Estimates tend to be low and non-significant, except in the more endemic taxa, and combined with measures of natural selection, suggest a higher potential for evolution in vegetative traits. Below we discuss the potential technical drawbacks of the analyses, and the implications of these results for trait evolution in *Aquilegia* populations.

Field-based estimation of heritabilities

Our *Aquilegia* study populations have the appropriate genetic structure, i.e. a significant variance in relatedness, crucial to apply Ritland's regression-based method to estimate heritability (Ritland 1996; Csilléry *et al.* 2006; Shikano 2008). This genetic structure is likely the consequence of the limited seed dispersal and some level of self-fertilization characteristic of *Aquilegia*, which lead to subpopulation structure even in small populations.

Even though the requirement of significant Var (r) is met, heritability estimates are noisy and often fall outside of the theoretical range ($0 < h^2 < 1$), making across-population comparisons unviable. Alternatives to Ritland's method, however, are not necessarily more appropriate for unpedigreed populations. A recent "pedigree-free animal model" approach requires a positive definite relatedness matrix (Frentiu et al. 2008), which is difficult to build from pairwise r estimates and no previous generation

413 information. Maximum likelihood-based alternatives rely on *a priori* assumptions of the
414 distribution of relatedness in the study populations (Mousseau et al. 1998), *i.e.* at least
415 some previous information on the population genealogical structure is necessary.
416 The accuracy of heritability estimates based on Ritland's method has been
417 questioned by several authors (Garant & Kruuk 2005; van Kleunen & Ritland 2005;
418 Rodríguez-Ramilo *et al.* 2007; Bouvet *et al.* 2008). For particular populations with
419 complete pedigrees and behavioural information, pairwise relatedness methods are
420 outperformed by pedigree-based methods (Thomas *et al.* 2002; Coltman 2005; Frentiu
421 *et al.* 2008; Van Horn *et al.* 2008). This can be caused, at least in part, by the lack of
422 variance in relatedness in many populations, particularly in obligate outbreeders
423 (Csilléry et al. 2006). In any case, relatedness estimates tend to have large variances
424 (Lynch & Ritland 1999; Ritland 2000) and a very high number of microsatellite loci or
425 genotyped individuals might be needed for precise heritability estimates. Even if
426 regression-based h^2 estimates were not accurate, they still can be used for within-
427 population comparison of traits, as we do here (Klaper *et al.* 2001; Garant & Kruuk
428 2005; Bessega *et al.* 2009).
429
430 *Evolutionary potential of floral and vegetative traits*
431 In a review study considering more than 900 estimates of genetic correlations
432 Ashman and Majetic (2006) found that floral traits tend to be correlated to each other
433 and not to vegetative traits. Although we were not able to estimate genetic correlations
434 between traits for *Aquilegia*, phenotypic correlations show the same pattern and suggest
435 that variation in floral and vegetative traits in our study populations is decoupled and
436 can be analyzed separately. For both types of traits we detected low h^2 values, which is
437 often the case in natural conditions (Schoen *et al.* 1994; Conner *et al.* 2003; Winn 2004

438 Blows & Hoffmann 2005), and appears to be also common for floral traits in self-
439 compatible species (Ashman & Majetic 2006). This later trend could be explained by
440 low genetic variation caused by inbreeding, but the low h^2 values in natural settings can
441 be in turn the consequence of high environmental variance rather than low additive
442 genetic variance. The estimation of h^2 directly from uncontrolled environments can
443 confound the two sources of variance. In our analysis, however, we found no evidence
444 of local environmental differences that were associated with relatedness (r_e correlations
445 were non-significant) within populations, and by avoiding across-population
446 comparisons there is a smaller chance that our estimates are overwhelmed by
447 environmentally-related variance.
448 Our results suggest that floral and vegetative traits do not differ in their heritabilities,
449 and that therefore, their current differential ability to respond to selection depends on
450 the nature of selective pressures. Of course, contemporary evolvability does not
451 necessarily reflect past response to selection, because heritabilities can change (for
452 example, after strong events of selection or in variable environments, as in Wilson *et al.*
453 (2006) and selection regimes are well-known to vary in time (Clegg et al. 2008;
454 Siepielski et al. 2009). Our present analysis only considered selection gradients for one
455 reproductive season and on one fitness trait (seed production), which gives us only a
456 snapshot of how selection is acting in these populations. In addition, we were not able to
457 detect selection on specific traits, because we ran the analysis on composite floral and
458 vegetative variables. However, our global finding that vegetative traits currently have a
459 higher evolutionary potential than floral traits in *Aquilegia* is consistent with previous
460 evidence and strengthens the higher relative importance of vegetative adaptation over
461 floral adaptation in the radiation of the Iberian lineage.

462 First, even though floral characteristics do vary across *Aquilegia* species and even
463 among populations within species (Table 1), only vegetative traits differentiate taxa
464 significantly (Medrano et al. 2006). Compared to the North American lineage of the
465 genus, flowers of European species are much more uniform in form (pendent and short-
466 spurred) and color (blue or purple). The six floral traits in our analyses included both
467 traits potentially involved in pollinator attraction (sepal and petal dimensions) and
468 nectar spur characteristics, which have been shown to affect pollinator behaviour and
469 pollen transfer in North American *Aquilegia* (Fulton & Hodges 1999). We found no
470 consistent selection on these traits in this study, which is in accordance with what we
471 know about pollinators. European columbines have not been in contact with
472 hummingbirds (Bastida et al. 2010), and even though we have occasionally observed
473 hawkmoths visiting their flowers, lepidopteran pollinators are not important as they are
474 in North American species. Bumblebees and other bees are the main pollinators of all
475 Iberian species (unpublished results), and even though the specific taxa and relative
476 importance of floral visitors can vary among populations (Medrano et al. 2006), it
477 seems unlikely that their behavioural and morphological differences could promote
478 floral differentiation. In fact, a preliminary analysis of divergent selection on floral traits
479 in our 15 study populations did not find evidence of pollinator-mediated selection
480 (results not shown).
481 Second, Alcántara et al. (2010) found strong evidence of divergent selection on
482 inflorescence height, number of leaves and number of flowers per inflorescence in our
483 study populations, most likely as the result of adaptation to different elevations and the
484 amount of soil rockiness. Inflorescence height and number of flowers could also be
485 considered as attraction traits for pollinators, and the former is actually consistently
486 correlated with floral traits (see Table S4, Supporting information). However, along

with the number of leaves, both traits also reflect plant size and its associated physiological costs. Alcántara et al. (2010) found that they are negatively correlated with the amount of rocks in the soil, and therefore with water availability, and in consequence smaller plant sizes are found in rocky habitats. This association could be explained by phenotypic plasticity, but a common garden study with the same four taxa found low plasticity in vegetative traits in response to soil depth (Bastida 2009). The differentiation between taxa might be better explained by genetic differentiation, and the low h^2 values we found for the same traits in this study are expected if selection has been sustained through time.

Finally, our heritability results from two widespread subspecies and their endemic sister subspecies are interesting, because differentiation among them is presumably occurring at present. We found significant heritabilities much more often in populations of the narrowly-restricted subspecies (*A. v. nevadensis* and *A. p. cazorlensis*), than in their widespread relatives (*A. v. vulgaris* and *A. p. pyrenaica*). One possible explanation is that within-population variance due to environment is higher in the later populations. However, the low environmental correlations in our analyses do not support this possibility. The low heritabilities instead suggest that genetic variation has been purged of the widespread species in their more stable environments, while the narrowly-distributed species still harbour genetic variance. Yet local differentiation of floral traits in response to selection at the different populations of the endemic taxa is hardly expected, because significant selection on floral traits was detected only in one of the eight populations.

Concluding remarks

Columbine populations in our study are restricted to humid or shady environments, and during the hot and dry summers, these habitats can be seen as islands surrounded by inhospitable land. Under these conditions, selective pressures associated with different habitats are strong on vegetative traits and can lead to diversification aided by the isolation of individual populations (Bastida et al. 2010). It is likely that pollinator-mediated selection has likely changed little since the arrival of the first *Aquilegia* ancestors from Asia and, as a consequence, floral traits have had a minor role in the radiation of this part of the genus. The examination of current ability to respond to selection of floral and vegetative traits in multiple populations of columbines is consistent with this model, even if current estimates of heritability are not quantitatively accurate.

This study has exemplified a valuable use of Ritland´s marker-based method of inferring heritabilities directly in wild populations, for cases where the within-population comparison of genetic parameters is the focus of interest. While we wait for more powerful statistical computations and highly informative markers to estimate population genetics in wild populations (e.g. whole-genome assessment of relatedness, see Herrera and Bazaga 2009), Ritland and related methods remain a good option for long-lived plants (Andrew et al. 2005), and as we show here, for small populations with genetic substructure, which is common for many species.

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686

687 **Figure legends**

688 Fig. 1. Estimates of mean relatedness for 13 *Aquilegia* populations and its relation to
689 approximate population size.

690 Table 1. Study populations and number of individuals characterized for this study.

691

Taxon	Population	Location	UTM coordinates		Population size	Sample size
			East	North		
Aquilegia v.	B. Jabalises	Sierra de Segura	30S 536356	4228894	80	42
vulgaris	Garrotegordo	Sierra de Segura	30S 533550	4229313	27	21*
	F. Reina	Sierra de Cazorla	30S 514740	4199585	115	50*
	S. Cabrilla	Sierra de Cazorla	30S 518770	4197610	138	33
Aquilegia v.	F. Fría	Sierra Nevada	30S 456428	4097019	120	50
nevadensis	Pradollano	Sierra Nevada	30S 464349	4105811	213	44
	Cortijuela	Sierra Nevada	30S 457931	4103212	71	37
	S. Maroma	Sierra Tejeda	30S 408767	4085378	60	45
Aquilegia p.	Tortiellas	Pyrenees	30T 700972	4739335	110	50
pyrenaica	Tobazo	Pyrenees	30T 701597	4739703	350	52
	Larra	Pyrenees	30T 679687	4758837	130	46
Aquilegia p.	B. Canal	Sierra de Cazorla	30S 503431	4182541	147	44
cazorlensis	Cabañas	Sierra de Cazorla	30S 503820	4184903	77	60
	C. del Aire	Sierra de Cazorla	30S 512371	4200647	156	50
	B. Charca	Sierra de Cazorla	30S 511977	4199404	267	46

692 * Populations excluded from molecular analysis due to low sample size or allelic variation

693 Table 2. Phenotypic differences among *Aquilegia* taxa and populations in the six
694 measured floral traits. Differences were tested using univariate linear models for taxa,
695 and mixed model tests for population as a nested factor. All tests are significant after
696 Bonferroni correction.
697

	Taxon			Population (Taxon)	
	F	d.f	P	LL ratio	P
Sepal length	147.73	3, 671	<0.001	13.099	0.004
Sepal width	504.18	3, 663	<0.001	29.090	<0.001
Spur width above nectary	147.76	3, 678	<0.001	26.182	<0.001
Spur aperture	93.53	3, 667	<0.001	12.260	0.007
Spur length	310.84	3, 669	<0.001	24.825	<0.001
Petal blade length	383.91	3, 678	<0.001	28.039	<0.001

698

699 Table 3. Estimated within-population variance in relatedness (*Var r*) and heritability values (h^2) for six floral and six vegetative traits in 13 wild
700 populations of *Aquilegia*. Estimates in bold are significantly positive with * P < 0.1, ** P < 0.05, *** P < 0.01, except *Var r* estimates which are
701 all significant at P < 0.001.

			Floral traits						**Vegetative traits**					
Taxon	**Population**	***Var r***	Sepal length	Sepal width	Spur width a. nectary	Spur aperture	Spur length	Petal blade length	Inflo. Height	Num. Leaves	Leaf Length	Num. Flowers	SLA	Nongland. Pub.
A. v.	B. Jabalises	0.002	0.302	-0.054	-0.664	-0.226	-0.22	-0.12	0.945	0.217	0.725	0.105	-0.779	1.176
vulgaris	S. Cabrilla	0.005	0.311	-0.182	1.106	0.038	0.101	-0.137	-0.078	0.895	-0.234	1.231	-0.379	0.158
A. v.	F. Fría	0.014	**0.326***	-0.024	0.074	-0.016	0.153	-0.047	-0.047	-0.127	0.025	0.167	-0.04	0.25
nevadensis	Pradollano	0.009	0.243	0.245	0.274	0.011	-0.036	**0.835****	-0.101	0.014	-0.176	0.01	0.253	0.249
	Cortijuela	0.03	**0.435***	**0.772****	0.214	0.108	**0.352***	**0.510****	0.049	-0.119	-0.149	0.018	**0.338***	-0.144
	S. Maroma	0.018	**0.362***	0.039	0.098	-0.005	**0.357***	-0.107	0.028	0.099	0.013	0.063	-0.035	-0.039
A. p.	Tortiellas	0.019	0	-0.054	-0.133	0.086	-0.027	0.061	0.045	-0.065	0.043	0.05	0.07	0.109
pyrenaica	Tobazo	0.016	-0.071	-0.141	0.049	-0.051	-0.12	-0.027	-0.007	0.067	-0.074	0.001	0.093	-0.096
	Larra	0.004	**1.003****	0.37	0.398	0.692	0.407	0.335	0.657	0.906	0.175	-0.165	0.12	-0.027
A. p.	B. Canal	0.009	0.25	0.523	**0.893****	-0.039	**0.588***	0.046	-0.222	0.114	-0.171	0.268	-0.194	-0.261

cazorlensis	Cabañas	0.006	**1.561*** **	**0.707** **	-0.154	0.303	**0.967***	-0.077	**4.032*** **	**0.709***	**4.081*** **	**1.817*** **	-0.154	**0.851***
	C. del Aire	0.004	0.43	0.157	0.464	0.003	-0.138	0.43	**1.08** **	0.161	**0.908** **	-0.157	0.302	**0.862***
	B. Charca	0.004	0.335	0.392	0.51	0.27	0.576	0.347	0.237	0.102	0.251	-0.081	**0.704***	0.128

Table 4. Selection gradients on composite floral and vegetative variables for each population. The variables are the main factors (PCs) of separate principal components analyses on floral and vegetative traits. Values in bold are significant with P<0.05.

		Floral factors		Vegetative factors	
Taxon	**Population**	**PC1**	**PC2**	**PC1**	**PC2**
A. v. vulgaris	Fte. Reina	-0.064	0.046	-0.179	0.231
	S. Cabrilla	0.117	-0.013	-0.342	0.009
A. v. nevadensis	F. Fría	0.099	-0.063	**-0.514**	0.153
	Pradollano	-0.002	0.004	-0.101	**0.755**
	Cortijuela	-0.160	0.030	-0.088	**0.577**
	S. Maroma	0.186	-0.069	**-0.510**	0.168
A. p. pyrenaica	Tortiellas	-0.273	-0.043	-0.099	-0.005
	Tobazo	-0.187	0.247	**-0.349**	**0.453**
	Larra	-0.165	0.203	-0.091	**0.885**
A. p. cazorlensis	B. Canal	-0.133	0.012	**-0.569**	0.315
	Cabañas	**0.681**	-0.213	**-0.701**	0.208
	C. del Aire	-0.244	0.132	-0.307	0.155
	B. Charca	-0.261	-0.110	**-0.335**	0.293

Table 5. Estimated within-population heritability values (h^2) and the average environmental correlation (r_e) for two floral and two vegetative principal components factors (PCs) in *Aquilegia* populations. Estimates in bold are significantly positive with * P < 0.1, ** P < 0.05, *** P < 0.01.

		floral				**vegetative**			
		PC-1		**PC-2**		**PC-1**		**PC-2**	
Taxon	**Population**	h^2	r_e	h^2	r_e	h^2	r_e	h^2	r_e
A. v. vulgaris	S. Cabrilla	-0.80	0.10	-0.34	0.01	-0.53	0.05	0.11	-0.07
A. v.	F. Fría	-0.01	-0.02	-0.07	-0.01	-0.04	-0.02	0.22	-0.09
nevadensis	Pradollano	0.53	-0.15	0.00	-0.02	0.08	-0.05	-0.33	0.06
	Cortijuela	**0.46****	-0.17	**0.31***	-0.12	-0.07	-0.01	-0.16	0.02
	S. Maroma	0.23	-0.11	0.08	-0.06	-0.04	-0.01	-0.17	0.04
A. p.	Tortiellas	0.04	-0.04	0.02	-0.03	-0.05	-0.01	-0.03	-0.02
pyrenaica	Tobazo	-0.07	0.00	-0.04	-0.01	-0.02	-0.02	0.05	-0.04
	Larra	**0.96***	-0.18	**1.18****	-0.21	-0.04	-0.02	1.20	-0.21
A. p.	B. Canal	-0.22	0.02	0.11	-0.05	-0.49	0.08	**0.41***	-0.12
cazorlensis	Cabañas	**1.39****	-0.24	0.23	-0.06	**4.41*****	-0.72	0.55	-0.11
	C. del Aire	0.11	-0.04	0.09	-0.03	**0.87****	-0.15	0.06	-0.031
	B. Charca	0.37	-0.08	0.42	-0.08	0.66	-0.12	0.09	-0.04

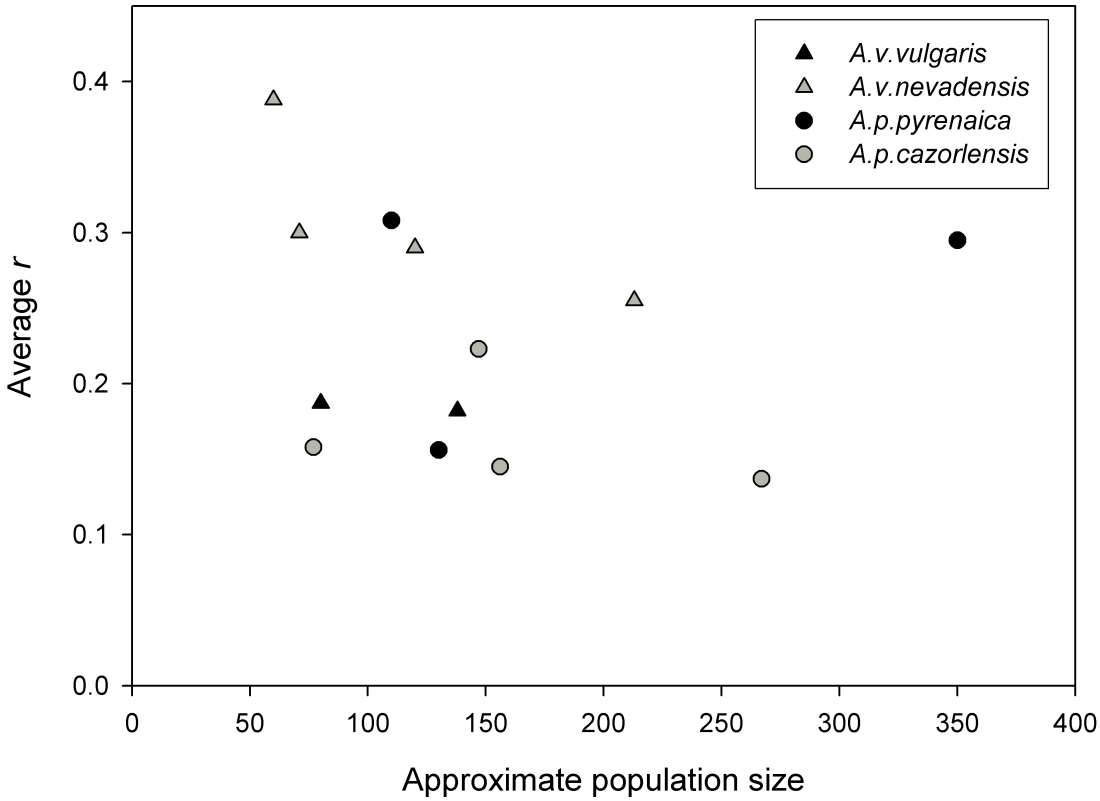
A.v.vulgaris
A.v.nevadensis
A.p.pyrenaica
A.p.cazorlensis
Average r
0.0
0.1
0.2
0.3
0.4
0
50
100
150
200
250
300
350
400
Approximate population size

Table S1. Allele richness in 13 *Aquilegia* populations for 10 microsatellite loci taken from Yang *et al*. (2005), where primer sequences can be obtained.

	Population												
	A. v. vulgaris		*A. v. nevadensis*				*A. p. pyrenaica*			*A. p. cazorlensis*			
Locus	**JAB**	**CLL**	**D**	**PLL**	**COR**	**MAR**	**TOR**	**CTT**	**LAR**	**CAN**	**Cab**	**COV**	**CHA**
7-27.1	10	11	2	5	4	6	3	2	5	6	8	5	6
25.3-33	4	3	5	4	6	4	6	7	5	1	1	5	4
50-21	6	2	2	2	2	2	2	1	1	5	7	3	5
10-15	6	3	2	4	3	3	1	1	1	NA	NA	3	3
1-40	4	3	4	2	3	1	2	2	3	2	1	4	2
13-39	1	1	2	1	1	2	2	1	6	2	2	1	1
50-9	1	1	6	2	4	2	2	2	3	1	1	1	1
50-7	2	4	2	4	4	2	2	2	3	1	2	3	3
7-27.2	3	9	8	7	2	1	9	7	16	8	8	15	19
25.6-16	3	3	2	4	2	2	2	2	3	2	6	8	7
Total number of alleles	40	40	35	35	31	25	31	27	46	28	36	48	51
Mean number of alleles	4	4	3.5	3.5	3.1	2.5	3.1	2.7	4.6	3.111	4	4.8	5.1

Populations: JAB=B. Jabalises, CLL= S. Cabrilla, D= F. Fría, PLL=Pradollano, COR=Cortijuela, MAR=S. Maroma, TOR=Tortiellas, CTT=Tobazo, LAR=Larra, CAN=B. Canal, Cab=Cabañas, COV=C. del Aire, CHA=B. Charca.

Table S2. Correlations of phenotypic traits with the factors of principal component (PC) analysis based on all populations in this study. Traits were log-transformed, except for number of leaves and number of flowers per inflorescence, which were square root-transformed. A. Floral traits. B. Vegetative traits.

A.

	PC1	PC2	PC3	PC4	PC5	PC6
Sepal length	**-0.919**	-0.124	0.066	0.241	0.099	-0.259
Sepal width	**-0.780**	0.426	0.334	0.223	0.051	0.215
Spur width a. nectary	-0.093	**-0.847**	0.497	-0.070	-0.144	0.017
Spur aperture	-0.601	**-0.671**	-0.330	-0.061	0.241	0.134
Spur length	**-0.872**	-0.027	-0.336	0.012	-0.353	0.034
Petal blade length	**-0.794**	0.361	0.157	-0.457	0.058	-0.051
Eigenvalue	3.213	1.496	0.610	0.326	0.219	0.136
% of total variance	53.55	24.94	10.17	5.43	3.66	2.26

B.

	PC1	PC2	PC3	PC4	PC5	PC6
Inflo. Height	**-0.954**	-0.050	-0.051	-0.137	0.127	0.223
Num. Leaves	-0.136	**0.831**	0.502	0.194	0.009	0.027
Leaf Length	**-0.879**	-0.013	0.250	-0.280	0.243	-0.163
Num. Flowers	**-0.830**	0.226	-0.250	-0.156	-0.415	-0.044
SLA	-0.414	**-0.556**	0.661	0.229	-0.175	0.007
Nongland. Pub.	**-0.723**	-0.017	-0.423	0.531	0.111	-0.054
Eigenvalue	3.085	1.054	0.995	0.494	0.290	0.082
% of total variance	51.42	17.57	16.58	8.23	4.84	1.37

Table S3. Mean population values for the six floral traits measured. See Table 1 for sample sizes. See Alcántara *et al.* for an equivalent table of vegetative traits.

		Floral traits					
Taxon	**Population**	Sepal length	Sepal width	Spur width above nectary	Spur aperture	Spur length	Petal blade length
A. v.	B. Jabalises	21.78 ± 2.995	8.04 ± 1.282	1.51 ± 0.267	8.22 ± 1.337	12.01 ± 2.115	8.50 ± 1.429
vulgaris	Garrotegordo	25.78 ± 2.484	8.84 ± 1.479	1.41 ± 0.283	9.23 ± 1.114	14.18 ± 1.801	8.73 ± 1.104
	F. Reina	22.51 ± 2.205	10.56 ± 1.338	1.62 ± 0.284	7.51 ± 0.953	12.15 ± 1.486	9.71 ± 1.442
	S. Cabrilla	22.56 ± 2.622	9.91 ± 1.058	1.33 ± 0.212	7.64 ± 1.029	10.49 ± 1.824	11.51 ± 1.207
A. v.	F. Fría	20.20 ± 2.182	6.28 ± 0.984	1.30 ± 0.231	8.09 ± 0.892	14.45 ± 1.917	10.60 ± 0.989
nevadensis	Pradollano	26.21 ± 3.663	8.80 ± 1.208	1.61 ± 0.356	8.87 ± 1.054	15.21 ± 1.790	11.82 ± 1.928
	Cortijuela	32.15 ± 3.456	10.61 ± 1.646	1.49 ± 0.247	9.69 ± 0.937	17.61 ± 1.872	11.80 ± 1.137
	S. Maroma	27.52 ± 3.502	9.42 ± 1.207	1.33 ± 0.324	9.31 ± 0.829	15.49 ± 2.190	9.62 ± 1.261
A. p.	Tortiellas	26.15 ± 3.563	13.97 ± 2.079	0.96 ± 0.128	6.80 ± 0.929	16.66 ± 1.586	13.73 ± 1.725

pyrenaica	Tobazo	29.42 ± 3.511	16.35 ± 2.062	0.98 ± 0.166	7.81 ± 0.699	18.37 ± 1.403	15.75 ± 1.850
	Larra	28.37 ± 4.591	16.02 ± 2.860	0.97 ± 0.167	7.38 ± 1.216	17.78 ± 2.387	15.53 ± 1.630
A. p.	B. Canal	20.16 ± 2.757	8.98 ± 1.482	1.13 ± 0.207	6.16 ± 0.858	10.89 ± 1.208	9.10 ± 1.168
cazorlensis	Cabañas	16.45 ± 2.540	7.76 ± 1.253	1.06 ± 0.243	6.52 ± 0.854	10.46 ± 1.487	9.70 ± 1.127
	C. del Aire	22.77 ± 2.628	7.29 ± 0.902	1.21 ± 0.196	8.36 ± 1.038	12.69 ± 1.267	9.91 ± 1.250
	B. Charca	19.26 ± 2.360	6.77 ± 1.076	1.18 ± 0.188	7.42 ± 0.872	13.31 ± 1.695	9.00 ± 1.054

Table S4. Phenotypic correlations among all floral and vegetative traits for one population of *Aquilegia vulgaris vulgaris* (B. Jabalises). The box highlights the correlations among floral and vegetative traits. All traits were ln-transformed for the tests. Asterisks indicate significant correlations: * P < 0.05, ** P < 0.01, *** P < 0.001

	Sepal length	Sepal width	Spur width a. nectary	Spur aperture	Spur length	Petal blade length	Inflo. Height	Num. Leaves	Leaf Length	Num. Flowers	SLA
Sepal width	0.63 ***										
Spur width a. nectary	0.45 **	0.21									
Spur aperture	0.82 ***	0.31 *	0.34 *								
Spur length	0.76 ***	0.37 *	0.51 ***	0.76 ***							
Petal blade length	0.71 ***	0.28	0.15	0.74 ***	0.37 *						
Inflo. Height	0.49 **	0.30	0.23	0.48 **	0.4 **	0.37 *					
Num. Leaves	-0.09	0.09	-0.08	-0.05	-0.16	0.13	0.12				
Leaf Length	0.16	0.29	0.17	0.11	-0.01	0.13	0.30	0.68 ***			
Num. Flowers	0.32 *	0.24	0.11	0.22	0.20	0.21	0.52 ***	0.26	0.06		
SLA	-0.14	0.02	0.11	-0.01	0.02	0.07	0.07	0.04	0.18	-0.48 **	
Nongland. Pub.	0.19	0.16	0.05	0.11	0.22	0.18	0.16	0.14	0.11	0.16	0.37 ***