

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

MARIA CLARA CASTELLANOS,\* JULIO M. ALCÁNTARA,† PEDRO J. REY† and JESUS M. BASTIDA†

\*Consejo Superior de Investigaciones Científicas, Centro de Investigaciones sobre Desertificación (CSIC-UIV-GV), 46470 Albal, Valencia, Spain, †Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad de Jaén, 23071 Jaén, Spain

## 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.

**Keywords:** columbine, evolvability, heritability, microsatellites, pollinators, relatedness

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## 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 carried out. 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 (Shaw 1986; Riska *et al.* 1989). 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

Correspondence: Maria Clara Castellanos, Fax: +34 96 127 0967; E-mail: maclacas@uv.es

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 with 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; Andrew *et al.* 2005). In addition, controlled conditions are also inappropriate for plants with mixed mating systems that are difficult to emulate with manual pollinations. A third advantage of field methods is that they can be applied in studies that require large spatial or temporal scales that cannot be addressed with controlled crosses, such as to detect whether trait heritabilities change across years in a population, or to estimate genetic parameters in multiple populations as required by studies of character divergence. It is therefore important to test marker-based methods and their applicability to different plant populations.

Analytical tools have been developed for inference of quantitative genetic parameters for field data sets both with partial or complete pedigrees, and for the estimation of relatedness with no previous knowledge or assumptions about a population's genealogical structure (reviewed by Garant & Kruuk 2005). In principle, the reconstruction of a pedigree is best for the estimation of quantitative genetic parameters in a natural population (e.g. the 'animal model'; Thomas *et al.* 2002; Garant & Kruuk 2005). Pedigree reconstruction methods require at least some previous information, such as discriminating adults from offspring, or behavioural observations of possible matings. Even with such information, marker-based pedigree reconstruction methods for wild populations are not perfect (Butler *et al.* 2004), as they are sensitive to data errors and mutations. Furthermore, under some circumstances pedigrees might not even be desirable. For many long-lived plant species where individuals can self-pollinate and generations are indistinguishable in the field, the reconstruction of a pedigree is not only troublesome, but it is likely that a continuous measure of relatedness is even a better representation of relationships.

For such plant populations, one pedigree-free alternative for estimating heritabilities is Ritland (1996)'s regression-based method, which relies on pairwise relatedness estimates and phenotypic similarity to estimate heritability of the traits of interest through linear regression. Several marker-based estimators of related-

ness have been developed (reviewed by Blouin 2003), and although they are not error-free, they can be used in Ritland's regression approach in combination with an estimation of the actual variance in relatedness [ $\text{Var}(r)$ ] in the population. The method requires that populations harbour significant  $\text{Var}(r)$  (i.e. that there is a mixture pairs of individuals related to various degrees), which is one of the major obstacles for its application to wild populations (Ritland 1996; Csilléry *et al.* 2006; Shikano 2008).

In tests involving populations of obligate outbreeders where authors have compared  $h^2$  values estimated by Ritland's method and other pedigree-based methods, Ritland's estimates are often found to be inaccurate (Thomas *et al.* 2002; Coltman 2005; Frentiu *et al.* 2008; Van Horn *et al.* 2008; but see DiBattista *et al.* 2008). However, outbred vertebrates often present low variance in relatedness (Csilléry *et al.* 2006). In other organisms, evidence that Ritland-based estimations can be reasonably accurate is slowly accumulating (Andrew *et al.* 2005; DiBattista *et al.* 2008; Herrera & Bazaga 2009; Anderson *et al.* 2010). Andrew *et al.* (2005), for instance, successfully estimated heritabilities of defence chemicals in a population of a long-lived tree species and argued that, provided that there is enough variance in relatedness, it can be a useful approach. Yet even if this requirement is met, estimation of heritabilities using molecular markers can suffer from low statistical power (Ritland 1996; Rodríguez-Ramilo *et al.* 2007), as is the case for the estimation of quantitative parameters with other methods (Mitchell-Olds & Rutledge 1986). Large sample sizes and highly polymorphic markers might be required, and this might have helped prevent the use of this method more extensively. However, small population size is the rule for many plant species. Here, we test the regression approach for small wild populations, aiming to make intrapopulation comparisons of trait heritabilities, rather than trying to compare estimates across taxa. Even if estimates are not accurate, and even outside of the theoretical range, we show that this approach can be useful when within-population comparisons are needed.

We estimated heritabilities of floral and vegetative traits in Iberian populations of the herb genus *Aquilegia* (Ranunculaceae) in order to compare the ability to respond to selection of both types of traits within each population. In *Aquilegia*, two contrasting, recent radiations have occurred simultaneously in North America and Europe, after the colonization by an Asian ancestor, and both have given rise to about the same number of species (Bastida *et al.* 2010). In the New World, the diversification of the group has been associated with floral adaptation to different pollinators (Grant 1952; Hodges & Arnold 1995). Specialized floral morphologi-

cal features, mainly the elongated petals that form a nectariferous spur, help determine the identity of the pollinators that can reach the nectar and lead to rapid floral specialization on bees, hummingbirds or moths, and ultimately to reproductive isolation (Whittall & Hodges 2007). Even though habitat characteristics also differ between North American *Aquilegia* species (Chase & Raven 1975; Hodges & Arnold 1994), the major role of pollinators is evident because shifts to different pollinators are common in the phylogeny (Whittall & Hodges 2007) and recent speciation events may have occurred in sympatry (Bastida *et al.* 2010). In contrast, pollinator shifts are absent from the European lineage (all species are pollinated by bumblebees and other bees), while habitat shifts have been common (Bastida *et al.* 2010). In addition, at least in some groups, vegetative traits are more important than floral traits in differentiating species (Medrano *et al.* 2006). A recent study further shows that vegetative traits in Iberian columbines have diverged in response to 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 vs. 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

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

Taxon	Population	Location	UTM coordinates		Population size	Sample size
			East	North		
<i>A. v. vulgaris</i>	B. Jabalises	Sierra de Segura	30S 536356	4228894	80	42
	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
<i>A. v. nevadensis</i>	F. Fría	Sierra Nevada	30S 456428	4097019	120	50
	Pradollano	Sierra Nevada	30S 464349	4105811	213	44
	Cortijuela	Sierra Nevada	30S 457931	4103212	71	37
	S. Maroma	Sierra Tejada	30S 408767	4085378	60	45
<i>A. p. pyrenaica</i>	Tortielas	Pyrenees	30T 700972	4739335	110	50
	Tobazo	Pyrenees	30T 701597	4739703	350	52
	Larra	Pyrenees	30T 679687	4758837	130	46
<i>A. p. cazorlensis</i>	B. Canal	Sierra de Cazorla	30S 503431	4182541	147	44
	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

\*Populations excluded from molecular analysis because of low sample size or allelic variation.

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 of 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 SIGMA SCAN 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 flowers per inflorescence, specific leaf area, and density of non-glandular pubescence in leaves. Specific leaf area was determined in the laboratory from a sample of the longest leaf, and the density of pubescence in the leaves was estimated under dissection binoculars from fresh epidermal tissue (details in Alcántara *et al.* 2010). In addition, seed production of each individual plant was estimated as the product of (i) the number of healthy seeds produced in a single fruit collected in the field and (ii) the total number of ripe carpels produced by the plant.

#### *DNA extraction and microsatellite analysis*

DNA of each individual plant was extracted from dried leaf material using the Speedtools Plant DNA Extraction Kit (Biotools, Madrid, Spain). We amplified 10 microsatellite loci, chosen among those developed for North American *Aquilegia* by Yang *et al.* (2005), with some

modifications on the PCR protocol. For 20- $\mu$ L PCRs, we added template DNA, 0.25  $\mu$ M of each primer (forward primers were labelled with fluorophores), 0.1 mM of each dNTP, 1 unit of Taq polymerase, 3.5 mM of MgCl<sub>2</sub>, and 1 $\times$  reaction buffer. PCR started with a 4-min denaturation phase at 94 °C, followed by 38 cycles of 94 °C, 45 s; 56–62 °C, 45 s; 72 °C, 45 s, and a final extension step of 72 °C for 10 min. Fragment analysis was carried out on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). We visualized peaks with GENEMAPPER Software v.4.0 (Applied Biosystems) and used MstatAllele in R (Alberto 2009) combined with manual checking for allele scoring. One locus (10–15) failed to amplify in two *Aquilegia p. cazorlensis* populations (Table S1, Supporting information).

For each genotyped population, we checked for linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium in GENEPOP 4.0 (Rousset 2008). There was no evidence of LD, as no pair of loci showed a consistent correlation across populations within each species. Homozygote excess compared with 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. 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

[ $\text{Var}(r)$ ] for all populations, when compared with Queller & Goodnight (1989), Lynch & 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  $\text{Var}(r)$ .

#### 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 because of shared environment and  $e_{ij}$  is the error. Incorporating a correlation because of 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

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

where  $\text{Var}(r_{ij})$  is the actual variance in relatedness, a population parameter that needs to be high for this method to work.  $\text{Var}(r_{ij})$  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 re-sampled. 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  $\text{Var}(r_{ij})$ . Because 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  $\text{Var}(r_{ij})$  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 & Arnold (1983). PCs were standardized to zero mean and unit variance and used to estimate directional selection gradients ( $\beta$ ) 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 subspecies and also all populations within subspecies differ from each other in all six floral traits measured (Table 2), as they do for six vegetative traits (Alcántara *et al.* 2010).

We estimated phenotypic correlations among In-transformed traits for each population. The actual pairs of traits that are significantly correlated vary from one population to another, but two general patterns hold for all 15 populations (see Table S4, Supporting information, for the correlation matrix of one *A. v. vulgaris* population, Jabalises, as an example): floral traits are more likely to be correlated among themselves than vegetative traits, and floral and vegetative traits are only occasionally correlated. For all populations, on average, 10 ( $\pm 2.9$ ) of the 12 possible correlations among floral traits are significant (and positive), while 4.2 ( $\pm 1.5$ ) of 12 of vegetative traits are. Finally, 7.6 ( $\pm 5.1$ ) of the 36 possible correlations between floral and vegetative traits are significant and can be either positive or negative.

### Relatedness

Two *A. v. vulgaris* populations (Garrotegordo and F. Reina) with very low allelic variation were excluded from the molecular analyses, because error estimation of relatedness increases highly in such cases. In general, we found high values of mean pairwise relatedness,  $r$ ,

for the remaining 13 populations, which varied from 0.137 and 0.388 and were uncorrelated with population size (Fig. 1; Pearson  $r_p = -0.14$ ,  $P = 0.65$ ). Relatedness was particularly high for *A. v. nevadensis* populations. The variance in relatedness,  $\text{Var}(r)$ , was significantly greater than zero in all populations and ranged between 0.002 and 0.03.

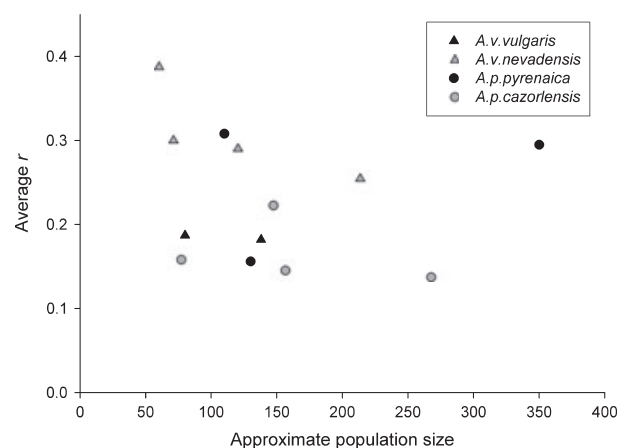
### Heritability

The significant values of  $\text{Var}(r)$  allowed us to test for statistical significance of heritability estimates for all traits in 13 populations. Most traits in most populations showed  $h^2$  values that were not significantly different from zero, with some exceptions (Table 3). Estimates of  $h^2$  outside of the theoretical range ( $0 < h^2 < 1$ ) are a consequence of Ritland's method and would represent a problem if we were interested in accurate  $h^2$  values, but in this case we focus on intra-population comparisons. Because of space limitations, Table 3 does not include the values for  $r_e$ , the correlation because of shared environment included in the model. This correlation was not significantly higher than zero in any of the traits or populations, indicating that there were no local environmental effects (i.e. patchiness) that differentially affected related individuals growing close to each other.

In general, vegetative and floral traits show similar  $h^2$  values: in a comparison of six floral vs. six vegetative traits for all populations using paired  $t$ -tests (which restrict comparisons to within-population), only 1 of the 36 contrasts was significant (sepal length vs. SLA). When comparing mean values of floral vs. vegetative traits within each population, we found that in only two populations, each of a different species, floral traits were more heritable when compared with vegetative traits of the same population ( $t = 2.306$ ,  $P = 0.028$  for B.

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

	Taxon			Population (taxon)	
	<i>F</i>	d.f.	<i>P</i>	LL ratio	<i>P</i>
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



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

**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 populations of *Aquilegia*

Taxon	Population	Floral traits						Vegetative traits						
		Var( $r$ )	Sepal length	Sepal width	Spur width above nectary	Spur aperture	Spur length	Petal blade length	inflorescence Height	Number of Leaves	Leaf Length	Number of Flowers	SLA	Non-glandular pubescence
<i>A. v. vulgaris</i>	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
	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
<i>A. v. neodarvensis</i>	F. Fría	0.014	<b>0.326*</b>	-0.024	0.074	-0.016	0.153	-0.047	-0.047	-0.127	0.025	0.167	-0.04	0.25
	Pradollano	0.009	0.243	0.245	0.274	0.011	-0.036	<b>0.835**</b>	-0.101	0.014	-0.176	0.01	0.253	0.249
<i>A. p. pyrenaica</i>	Cortijuela	0.03	<b>0.435*</b>	<b>0.772**</b>	0.214	0.108	<b>0.352*</b>	<b>0.510**</b>	0.049	-0.119	-0.149	0.018	<b>0.338*</b>	-0.144
	S. Maroma	0.018	<b>0.362*</b>	0.039	0.098	-0.005	<b>0.357*</b>	-0.107	0.028	0.099	0.013	0.063	-0.035	-0.039
<i>A. p. cazorlensis</i>	Tortillas	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
	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
<i>A. p. cazorlensis</i>	Larra	0.004	<b>1.003**</b>	0.37	0.398	0.692	0.407	0.335	0.657	0.906	0.175	-0.165	0.12	-0.027
	B. Canal	0.009	0.25	0.523	<b>0.893**</b>	-0.039	<b>0.588*</b>	0.046	-0.222	0.114	-0.171	0.268	-0.194	-0.261
<i>A. p. cazorlensis</i>	Cabañas	0.006	<b>1.561***</b>	<b>0.707**</b>	-0.154	0.303	<b>0.967*</b>	-0.077	<b>4.032***</b>	<b>0.709*</b>	<b>4.081***</b>	<b>1.817***</b>	-0.154	<b>0.851*</b>
	C. del Aire	0.004	0.43	0.157	0.464	0.003	-0.138	0.43	<b>1.08**</b>	0.161	<b>0.908**</b>	-0.157	0.302	<b>0.862*</b>
<i>A. p. cazorlensis</i>	B. Charca	0.004	0.335	0.392	0.51	0.27	0.576	0.347	0.237	0.102	0.251	-0.081	<b>0.704*</b>	0.128

Estimates in bold are significantly positive with \* $P < 0.1$ , \*\* $P < 0.05$ , \*\*\* $P < 0.01$ , except Var( $r$ ) estimates which are all significant at  $P < 0.001$ .

Canal and  $t = 3.27$ ,  $P = 0.008$  for Cortijuela). When focusing on the significance of heritability values only, a notable trend is that for the two widespread taxa, *A. v. vulgaris* and *A. p. pyrenaica*, there is essentially no trait with detectable heritability in any population. All but one of the heritable traits we could detect are in the more restricted, endemic subspecies.

*Phenotypic selection and ability to respond to selection*

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

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

**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.

**Table 4** Selection gradients on composite floral and vegetative variables for each population. The variables are the main factors of separate principal components (PCs) analyses on floral and vegetative traits

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

Values in bold are significant with  $P < 0.05$ .

Although our estimated  $h^2$  values are not directly comparable with 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. In the following paragraphs, 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  $\text{Var}(r)$  is met, heritability estimates are noisy and often fall outside of the theoretical range, 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 information. Maximum likelihood-based alternatives rely on *a priori* assumptions of the distribution of relatedness in the study populations (Mousseau *et al.* 1998), i.e. at least some previous information on the population genealogical structure is necessary.

**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

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

Estimates in bold are significantly positive with \* $P < 0.1$ , \*\* $P < 0.05$ , \*\*\* $P < 0.01$ .



The accuracy of heritability estimates based on Ritland's method has been questioned by several authors (Garant & Kruuk 2005; van Kleunen & Ritland 2005; Rodríguez-Ramilo *et al.* 2007; Bouvet *et al.* 2008). For particular populations with complete pedigrees and behavioural information, pairwise relatedness methods are outperformed by pedigree-based methods (Thomas *et al.* 2002; Coltman 2005; Frentiu *et al.* 2008; Van Horn *et al.* 2008). This can be caused, at least in part, by the lack of variance in relatedness in many populations, particularly in obligate outbreeders (Csilléry *et al.* 2006). In any case, relatedness estimates tend to have large variances (Lynch & Ritland 1999; Ritland 2000) and a very high number of microsatellite loci or genotyped individuals might be needed for precise heritability estimates. Even if regression-based  $h^2$  estimates were not accurate, they still can be used for within-population comparison of traits, as we do here (Klaper *et al.* 2001; Garant & Kruuk 2005; Bessega *et al.* 2009).

#### *Evolutionary potential of floral and vegetative traits*

In a review study considering more than 900 estimates of genetic correlations, Ashman & Majetic (2006) found that floral traits tend to be correlated with each other and not to vegetative traits. Although we were not able to estimate genetic correlations between traits for *Aquilegia*, phenotypic correlations show the same pattern and suggest that variation in floral and vegetative traits in our study populations is decoupled and can be analysed separately. For both types of traits, we detected low  $h^2$  values, which is often the case in natural conditions (Schoen *et al.* 1994; Conner *et al.* 2003; Winn 2004; Blows & Hoffmann 2005), and appears to be also common for floral traits in self-compatible species (Ashman & Majetic 2006). This later trend could be explained by low genetic variation caused by inbreeding, but the low  $h^2$  values in natural settings can be in turn the consequence of high environmental variance rather than low additive genetic variance. The estimation of  $h^2$  directly from uncontrolled environments can confound the two sources of variance. In our analysis, however, we found no evidence of local environmental differences that were associated with relatedness ( $r_e$  correlations were non-significant) within populations, and by avoiding across-population comparisons there is a smaller chance that our estimates are overwhelmed by environmentally related variance.

Our results suggest that floral and vegetative traits do not differ in their heritabilities, and that therefore, their current differential ability to respond to selection depends on the nature of selective pressures. Of course, contemporary evolvability does not necessarily reflect past response to selection, because heritabilities can

change (for example, after strong events of selection or in variable environments, as in Wilson *et al.* (2006) and selection regimes are well known to vary with time (Clegg *et al.* 2008; Siepielski *et al.* 2009). Our present analysis only considered selection gradients for one reproductive season and on one fitness trait (seed production), which gives us only a snapshot of how selection is acting in these populations. In addition, we were not able to detect selection on specific traits, because we ran the analysis on composite floral and vegetative variables. However, our global finding that vegetative traits currently have a higher evolutionary potential than floral traits in *Aquilegia* is consistent with previous evidence and strengthens the higher relative importance of vegetative adaptation over floral adaptation in the radiation of the Iberian lineage.

First, even though floral characteristics do vary across *Aquilegia* species and even among populations within species (Table 1), only vegetative traits differentiate taxa significantly (Medrano *et al.* 2006). Compared with the North American lineage of the genus, flowers of European species are much more uniform in form (pendent and short-spurred) and colour (blue or purple). The six floral traits in our analyses included both traits potentially involved in pollinator attraction (sepal and petal dimensions) and nectar spur characteristics, which have been shown to affect pollinator behaviour and pollen transfer in North American *Aquilegia* (Fulton & Hodges 1999). We found no consistent selection on these traits in this study, which is in accordance with what we know about pollinators. European columbines have not been in contact with hummingbirds (Bastida *et al.* 2010), and even though we have occasionally observed hawkmoths visiting their flowers, lepidopteran pollinators are not important as they are in North American species. Bumblebees and other bees are the main pollinators of all Iberian species (unpublished results), and even though the specific taxa and relative importance of floral visitors can vary among populations (Medrano *et al.* 2006), it seems unlikely that their behavioural and morphological differences could promote floral differentiation. In fact, a preliminary analysis of divergent selection on floral traits in our 15 study populations did not find evidence of pollinator-mediated selection (results not shown).

Second, Alcántara *et al.* (2010) found strong evidence of divergent selection on inflorescence height, number of leaves and number of flowers per inflorescence in our study populations, most likely as the result of adaptation to different elevations and the amount of soil rockiness. Inflorescence height and number of flowers could also be considered as attraction traits for pollinators, and the former is actually consistently 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 because of 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 & 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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The authors are interested in understanding the ecological and evolutionary drivers of plant biodiversity, and for that purpose combine field ecology, quantitative genetics and molecular ecology approaches. MCC focuses mainly on plant interactions. JMA, PJR and JMB are particularly interested in the Mediterranean Basin, one of the biodiversity hot spots in the planet.

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## Supporting information

Additional supporting information may be found in the online version of this article.

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

**Table S2** Correlations of phenotypic traits with the factors of principal component (PC) analysis based on all populations in this study.

**Table S3** Mean population values for the six floral traits measured.

**Table S4** Phenotypic correlations among all floral and vegetative traits for one population of *Aquilegia vulgaris vulgaris* (B. Jabalises).

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