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Genetic Drift Linked to Heterogeneous Landscape and Ecological Specialization Drives Diversification in the Alpine Endemic Columbine *Aquilegia thalictrifolia*

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Abstract

The European Alpine system is an extensive mountain range, whose heterogeneous landscape together with Quaternary climatic oscillations significantly affected organismal diversity and distribution in Europe. The model genus *Aquilegia* represents a textbook example of a rapid and recent radiation through the Northern hemisphere, with the majority of the European taxa occuring in the Alpine system. However, the processes governing genetic differentiation of the genus in this complex geographic area are still widely unexplored. In this work, we used 9 microsatellite loci to study the genetic structure and diversity of 11 populations of *Aquilegia thalictrifolia* Schott & Kotschy, an alpine taxon characterized by a marked ecological specificity. We found that, despite the endemic and fragmented distribution, *A. thalictrifolia* has overall high levels of heterozygosity, which is consistent to the substantial inbreeding depression that characterizes the genus. Strong spatial genetic structuring of populations suggests a historical prevalence of genetic drift over gene flow, with natural barriers and ecological niche hindering migration. An analytical comparison of fixation and population differentiation indexes allowed us to infer hypotheses of the postglacial history and more recent demographic events that have influenced the genetics of the species. Overall, our results indicate allopatry as a major force of differentiation in the European scenario, likely to underlie the development of taxonomic boundaries in a broader geographic context. This adds to previous notions on the primary evolutionary forces shaping the *Aquilegia* radiation in Europe.

Subject area: *Population structure and phylogeography*

Keywords: *allopatry, Alpine landscape, ecological niche, gene flow, population structure*

Genetic diversity of alpine taxa is shaped by a kaleidoscope of factors intrinsic to the heterogenous landscape that characterizes mountain environments. The European Alpine system, designating the mountain ranges that span from the Pyrenees to the Alps, the Balkans and the Carpathians, has been shown to constitute a major factor shaping organismal diversity in Europe at different spatial and temporal scales over a broad taxonomic range (Hewitt 2000; Ozenda 2009). Possibly, the clearest evidence of this process is imprinted at the lower level of biological diversity where populations constitute natural entities directly undergoing the effects of the natural environment. Ecological adaptation as a response to environmental conditions acts differently in populations in relation to their spatial distribution, in a dynamic process determined by the interplay of standing genetic variation and migration between populations adapted to contrasting

environments (Kremer et al. 2012). On the other hand, topographical features and climatic oscillations severely affect the geographic distribution of species, by triggering range shifts that, in turn, affect the genetic composition of populations. Quaternary climatic changes had a major impact on molding actual patterns of diversity in the European alpine system, often isolating fragmented populations in restricted areas and thus prompting speciation during glacial periods, while favoring expansion and admixture of genetically differentiated lineages during interglacials (Hewitt 2000; Petit et al. 2003). In this context, gene flow and genetic drift represent the main microevolutionary processes configuring the pattern of neutral genetic diversity at different hierarchical and spatial levels (Loveless and Hamrick 1984; Hartl 2000). The combined analysis of population structure and components of the landscape can provide insight on how these forces acted (Manel 2003) and unravel their relative roles in shaping discontinuities in genetic distribution (Hutchinson and Templeton 1999). This approach is particularly suited within the context of the heterogeneous alpine landscape, where a variety of physical barriers (mountains, forests, rivers, etc.) tend to limit gene flow. Additionally, the complex topography promotes differences in substrate, temperature, sun exposure, and moisture, thus creating an extraordinary variety of microclimates for local adaptation (e.g., Alvarez et al. 2009).

In recent years, *Aquilegia* (Ranunculaceae), also known as columbines, has become a model system in evolutionary studies, with prime findings on the genetics of speciation and floral development (Kramer 2009; Sharma et al. 2014). The genus is composed of approximately 70 species distributed in the Northern hemisphere (Munz 1946) and is defined as a "species flock" in light of the little phylogenetic signal accumulated in DNA sequences among species, which suggested a rapid process of diversification (Hodges and Arnold 1994a, 1995). Similarly, genetic variation at the species level has often proved insufficient to reflect taxonomic boundaries, even for entities diagnosed by discrete characters, including morphological features, ecological niches, and reproductive systems (Hodges and Arnold 1994b; Ro et al. 1997; Bastida et al. 2010; Cooper et al. 2010). In recent years, complementary efforts have been devoted to shed light on the nature of speciation underlying the apparently contrasting radiation of the columbines in the New and the Old Worlds. In their benchmark work on the evolution of North American species, Whittall and Hodges (2007) confidently showed that speciation in this group is strongly promoted by its pollination syndrome, with natural selection favoring flower spurs best matching tongue lengths of major pollinators. On the contrary, radiation in the Old World appears to be driven mostly by geographic isolation combined with habitat specialization (Bastida et al. 2010), with selection acting more on vegetative traits than on floral ones (Alcantara et al. 2010; Castellanos et al. 2011). Selective forces such as edaphic factors have been suggested to spur species diversification in relation to the shift from forests and meadows to a more saxicolous habitat (similar to current occurrence sites of Alpine species characterized by screes and rock crevices), typically during interglacial periods when mountainous regions remained free from the ice sheet (Bastida et al. 2010). According to this view, the saxicolous endemics presently distributed in Southern European Alps (e.g., *A. viscosa*, *A. thalictrifolia*, *A. pyrenaica*, *A. einseleana*, *A. bertolonii*) are to be considered stenoendemics (i.e., endemics restricted to a particular type of habitat) of recent origin.

Recently, Fior et al. (2013) produced a phylogeny of *Aquilegia* based on ~24Kb of the most rapidly evolving regions of the plastome, including multiple accessions for European taxa. Results from this work revealed that even amassing this amount of data could not provide sufficient information to resolve relationships within a large European clade, nor, in the majority of cases, to group multiple accessions of species in monophyletic groups. These results depict a complex scenario in which genetic patterns were likely shaped by repeated events of separation and introgression. In this context, the study of population genetics of European Alpine endemics can provide insights into the processes that regulate the distribution of genetic variation at a finer scale, relying on target infrageneric units that are regarded as well defined both taxonomically and geographically.

Aquilegia thalictrifolia Schott & Kotschy is a narrow endemic distributed in the Italian South-Eastern Alps, specifically on the Tremalzo–Tombea mountain chain. It is characterized by a glandulous indumentum and thick leaf pubescence that clearly distinguish it from the similar congener *A. einseleana* (Munz 1946; Akeroyd 1993). Only 22 populations are known, with less than 5000 mature ramets on a total distribution area of only 1443 m^2 (Bonomi et al. 2008). Its distribution range is characterized by a complex topography, which includes orographic discontinuities that separate groups of populations in different valleys. Most importantly, *A. thalictrifolia* is distinguished by a very specific ecological niche, as it inhabits calcareous bedrock in the proximity of water springs at the base of mountain cliffs, where dripping water keeps a constant level of moisture. This ecological specialization causes the fragmented distribution of the species and limits the number and size of existing populations. Moreover, this habitat appears to be particularly subject to increasing drought in virtue of the raising temperatures and decreasing precipitations due to climate change. For this reason, *A. thalictrifolia* has recently been classified as "Critically Endangered" at a global level (Bonomi et al. 2008).

The present work aims to study the population genetics of the alpine endemic *A. thalictrifolia* in order to gain insight on the distribution of the allelic variation within the species, and pinpoint the role of gene flow and genetic drift in shaping genetic structure. Comparative analyses relying on a traditional measure of fixation (G_{ST}) and an index of population differentiation (D_{est}) are used to generate hypotheses on the evolutionary history of *A. thalictrifolia* in the complex background of the landscape topology linked to its distribution. In this study, *A. thalictrifolia* provides a case study to address the underlying factors driving genetic differentiation in the Alpine system.

Materials and Methods

Sampling and DNA Extraction

A total of 295 individuals were sampled from 11 collection sites within 4 valleys representing the whole *A. thalictrifolia* distribution area (Bonomi et al. 2008; Figure 1): Valvestino (VE), Val d'Ampola (AM), Valle S. Michele (MI) and Val Pubregno (PU). Fresh and young leaves from 16 to 35 individuals per site were sampled. Because individuals often followed a patchy distribution, we took care to extensively explore each site and sample proportionally in all patches, choosing individuals 1–3 m apart within a patch. Due to the typical distribution at the base of rock cliffs, sampling often followed a 1-dimensional scheme. For every population, GPS coordinates were taken from the central point of the sampling, with an accuracy of 5 m on average. Detailed information about site locations and labels, number of individuals sampled per population, and voucher identification is provided in Table 1. Extraction of total genomic DNA was carried out using the Qiagen Dneasy 96 Well Plate Kit.

Simple Sequence Repeats Genotyping

Sixteen primer pairs for Simple Sequence Repeats (SSR) originally developed on North American *A. formosa* and *A. pubescens* by Yang et al. (2005) were tested on 2 individuals from 2 different sites of *A. thalictrifolia*. The 14 primer pairs that successfully amplified were further tested on a panel of 4 individuals from 4 sites to screen for polymorphic loci, and 9 proved variable among and/or within sites (i.e., 7–27.2, 200.2–4, 25.6–16, 50–7, 25.3–33, 11–3, 50–21, 10–15, 1–40; Yang et al. 2005). These were selected for subsequent genotyping (Table A, see Supplementary Material online).

Each locus was amplified independently in a reaction volume of 10 μL, containing 25 μM of each dNTP, 1× PCR buffer with $MgCl_2$ included, 0.02 μM of forward and reverse fluorescently labeled primers, 0.5 units of Taq DNA polymerase, and approximately 5–10 ng template DNA. The PCR program followed Yang et al. (2005) for all primers, except for 10–15 and 1–40, for which annealing temperature was set at 52 °C. One negative and one positive control were run in each of the 96-well plates used for amplification. For each individual, PCR products obtained with primers labeled with different fluorochromes were pooled in pairs and loaded on a 3730xl DNA Analyzer sequencer using the 1200 GeneScan LIZ as the internal size standard (Applied Biosystems, Carlsbad, CA). Fragment lengths were scored in Genemapper 4.0 software (Applied Biosystems) and manually assigned with a customized peak threshold (300–400 RFU) and binning (0.8–1.0 bp) on each allele. Ambiguous peaks were considered as missing data, in order to decrease genotyping errors due to stuttering and large allele dropout (Dewoody et al. 2006).

Descriptive Statistics

Linkage disequilibrium (LD) for each pair of loci in every site and across sites within the species was checked using the log-likelihood-ratio statistic in GENEPOP 4.1.4 (Rousset 2008). The Markov chain method was applied with 500 batches and 10 000 iterations per batch. Deviations from Hardy–Weinberg equilibrium (HWE) were also verified in GENEPOP both within and across sites, using the Probability-test (Haldane 1954; Guo and Thompson 1992; Weir 1996) and the score test (*U* test; Raymond and Rousset 1995), with the latter allowing to test both for heterozygote

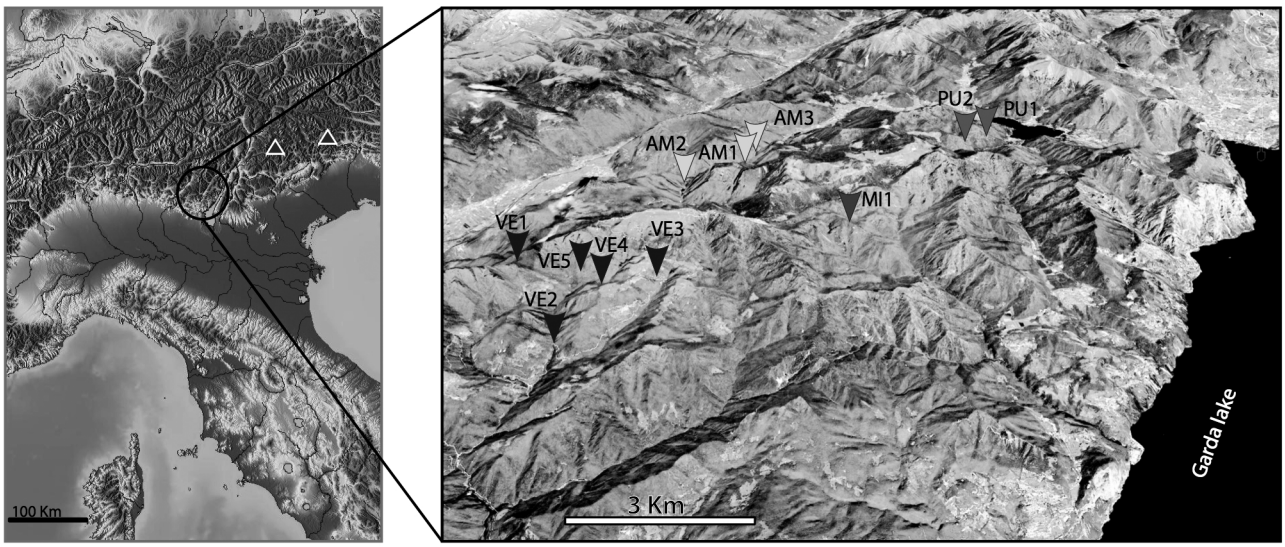

Figure 1. Geographic representation of the study area. (**A**) Part of the Italian Alpine system including the distribution range of *A. thalictrifolia* (circle) and the locations for the 2 hybrid populations with *A. einseleana* (triangles: see text for details). (**B**) Locations of the 11 collection sites for *A. thalictrifolia* within the 4 main drainages where the species is distributed. Collection site acronyms refer to Table 1.

Table 1 Sampling locations, identifications for the valleys where each site is located, collection site acronyms, number of individuals sampled per site, voucher, and genetic diversity estimates for each site across 9 microsatellite loci in *A. thalictrifolia* analyzed in this study

Sampling location (population ID)[a]	Voucher ID	No. samples	Hobs ±SD	Hexp ±SD	Mean ALoc ±SD	Ar	PAr
Bocca di Valle, Val Vestino (VE1)	ROV 61440	35	0.72 ± 0.18	0.74 ± 0.17	10.11 ± 5.51	7.3	1.16
Turano–Magasa, Val Vestino (VE2)	ROV 61397	23	0.70 ± 0.12	0.74 ± 0.12	6.67 ± 2.12	5.88	0.49
Loc. Pilaster, Val Vestino (VE3)	ROV 61401	30	0.61 ± 0.23	0.65 ± 0.25	8.33 ± 5.12	6.02	0.36
Loc. Ponte Franato, Val Vestino (VE4)	ROV 61395	28	0.63 ± 0.23	0.72 ± 0.24	11.11 ± 5.06	8.23	0.98
Messane, Val Vestino (VE5)	ROV 61438	27	0.75 ± 0.11	0.81 ± 0.15	12.00 ± 5.07	9.24	1.34
Rio Bragone, Val d'Ampola (AM1)	ROV 61400	20	0.65 ± 0.21	0.73 ± 0.15	7.78 ± 4.38	6.71	0.58
Val Lorina (AM2)	ROV 61514	32	0.67 ± 0.15	0.78 ± 0.14	10.89 ± 4.65	8.04	0.94
Tiarno di Sopra, Val d'Ampola (AM3)	ROV 61398	16	0.63 ± 0.19	0.72 ± 0.19	7.44 ± 3.91	7.04	0.99
Molina di Ledro, Val Pubregno (PU1)	N.A.	27	0.54 ± 0.31	0.57 ± 0.20	5.44 ± 5.27	4.55	1.44
Molina di Ledro, Val Pubregno (PU2)	TR s.n.	27	0.54 ± 0.32	0.50 ± 0.27	5.11 ± 4.37	4.35	0.68
Costa Monte di Mezzo, Valle S. Michele (MI1)	ROV 61399	30	0.54 ± 0.08	0.59 ± 0.10	4.22 ± 1.86	3.33	0.58
Mean ± SD			0.63 ± 0.19	0.68 ± 0.19	8.10 ± 4.30	6.43 ± 1.81	0.87 ± 0.36
Mean Htot ± SD			0.84 ± 0.13				

The voucher specimens were deposited in the herbarium collection of Civic Museum of Rovereto (ROV) and Museo di Scienze Naturali of Trento (TR) for future reference. Mean observed heterozygosity (Hobs); Nei's (1978) unbiased expected heterozygosity (Hexp); mean Hexp; mean total heterozygosity (Htot) across locations; mean number of alleles per locus (ALoc); allelic richness (Ar) and private allelic richness (PAr) adjusted for sample size (minimum sample size used for calculations = 24 genes). Standard deviations (SD) are provided for Hobs, Hexp, Mean ALoc, mean Hexp, and mean Htot. Collection site acronyms refer to Table 1.

[a]In the text, the populations from each valley are collectively indicated as follows: VE1–VE5 = VE; AM1–AM3 = AM; PU1–PU2 = PU; MI1 = MI.

deficiency and heterozygote excess. The Markov chain settings in the HWE tests were the same as in the LD analysis for loci with more than 5 alleles, whereas the complete enumeration method was applied for loci with up to 4 alleles per locus. When applicable, we checked for multiple testing by calculating the *P* values adjusted for the False Discovery Rate (FDR; Benjamini and Hochberg 1995) with the package fdrtool (Strimmer 2008) in R 2.15.1 (R Core Team 2012). When FDR was not applicable, we used Bonferroni correction. Finally, locus-by-population frequencies of null alleles were estimated with GENEPOP choosing the default estimation method of maximum likelihood based on the EM algorithm (Dempster et al. 1977).

Standard diversity indexes including observed heterozygosity (Hobs), expected heterozygosity (Hexp) and number of alleles averaged across loci (ALoc; Nei 1987) within every site, and mean total heterozygosity (Htot) across sites were calculated with ARLEQUIN 3.5. (Excoffier and Lischer 2010). Moreover, as small samples usually contain less alleles than large ones (Kalinowski 2004), unbiased measures of allelic richness (Ar) and private allelic richness (PAr) corrected for differences in sample size were estimated for each sampling site with the statistical technique of rarefaction implemented in HPRARE (Kalinowski 2005).

Population Structure

Microsatellite analyses were performed by applying the infinite allele model (IAM; Kimura and Crow 1964) to keep into account the observed departure from a stepwise increment of repeats in our data set.

As a preliminary analysis of population structure, a 3-level Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992; Michalakis and Excoffier 1996) was performed in ARLEQUIN. Analyses were conducted at the site and individual level, as well as within the 3 clusters recovered in the STRUCTURE analyses (corresponding to different "drainages" in which the species is distributed, see below). Significance of group partitioning was tested against alternative random distributions of individuals among groups through 10 000 random permutations. Differentiation between pairs of sites was assessed in ARLEQUIN calculating a global estimate across loci of the fixation index F_{ST} (Weir and Cockerham 1984), and its statistical significance was evaluated with 1000 random permutations. An overall estimate of Nei's G_{ST} (Nei 1973), an F_{ST} analogue for loci with multiple alleles, was also calculated for comparison.

Further analyses were performed to calculate D_{est} (Jost 2008), an estimator of actual differentiation corrected for small sample size, which is based on the effective number of alleles. In fact, the fixation indexes like F_{ST} and particularly G_{ST} can underestimate differentiation with highly polymorphic markers like microsatellites (Hedrick 2005; Jost 2008; Meirmans 2006). This limitation is overcome by D_{est}, which enables to separate whole genetic diversity into independent within- and between-deme components (Jost 2008, 2009).

The combined use of fixation- and differentiation-based measures is often recommended for a more exhaustive assessment of population structures (Meirmans and Hedrick 2011). Hence, overall and among-site values of both G_{ST} and D_{est} were calculated with the R package DEMEtics (Gerlach et al. 2010) and their confidence intervals (CIs) and associated *P* values were evaluated through 1000 bootstrap replicates with a Bonferroni correction for multiple testing. Correlations between G_{ST} and D_{est} measures were then verified through a Mantel test with 10 000 permutations

(Mantel 1967) implemented in the R Package Ade4 (Dray and Dufour 2007). As the 2 indexes quantify different aspects of population structure, we performed correlation analyses to test which model best approximated the relationship between them. For this purpose, the estimates of the parameters of a linear ($D_{est} = a * G_{ST}$) and an alternative nonlinear ($D_{est} = a * G_{ST} / (1 + b * G_{ST})$) model were determined with the function nls in R. Models were compared using the Akaike's Information Criterion (AIC). Moreover, as the 2 models are nested, analysis of variance (Anova) was also applied as a further confirmation of the chosen model. A graphical representation of the population structure described by G_{ST} and D_{est} and a visualization of any possible qualitative differences between the alternative measures was achieved through a classical multidimensional scaling (CMDS) plot computed on distance matrices based on both pairwise G_{ST} and D_{est} values, with the first 2 dimensions produced with the cmdscale function in R (R Core Team 2012).

The hypothesis of regional migration-drift equilibrium assuming a stepping-stone model of population structure (Kimura and Weiss 1964) was tested following the Hutchinson and Templeton method (1999). This approach evaluates the relative historical roles played by gene flow and genetic drift in shaping the structure of the populations under study, by testing the null hypothesis that both pairwise genetic distances and their variance increase monotonically with geographic distances (Hutchinson and Templeton 1999). Mantel tests (Mantel 1967) were applied both between genetic (linearized D_{est} and G_{ST}) and geographic distances, and between the variance in pairwise genetic and geographic distances (Hutchinson and Templeton 1999). The analyses were carried out both on a global scale and within the single clusters identified by Bayesian analyses in STRUCTURE (see below). Significance of correlations was estimated with 10 000 random permutations. Geographic distances were calculated with the R Package Sp (Pebesma and Bivand 2005; Bivand et al. 2008) as straight-line distances in kilometers between pairs of populations described by GPS coordinates. Mantel tests were executed with the R Package Ade4 (Dray and Dufour 2007).

To further test for increasing of the variance of genetic distances related to geographic distances we used generalized least squares (GLS) models using the function gls within the R package nlme (Pinheiro et al. 2013). We compared a linear regression model with constant variance (M0) with a model allowing increasing (M1) variance (by using the function varFixed) and chose the most likely one according to AIC.

A nonspatial Bayesian assignment method was adopted to determine populations based on genetic clusters and levels of admixture using STRUCTURE 2.3.3 (Pritchard et al. 2000). The model assumed admixture, correlated frequencies, and no prior population information. The following parameter settings were applied: 20 independent replicates each for a number of populations (K) ranging from K = 1 to K = 22 (i.e., twice the number of sites), a burnin period of 10^5 iterations, 10^5 subsequent Markov Chain Monte Carlo (MCMC) repetitions. Analyses were performed on the Bioportal server (http://www.bioportal.uio.no). The most likely number of populations was estimated with the ΔK statistic of Evanno et al. (2005) using STRUCTURE HARVESTER software (Earl and VonHoldt 2011). Multimodality in individual memberships coefficients and label switching across different runs were accounted for using the permutation procedure in CLUMPP (Jakobsson and Rosenberg 2007). The resulting matrix of Q values was graphically displayed through DISTRUCT (Rosenberg 2004).

Finally, a spatial analysis was carried out on the georeferenced genetic data using the extension for R of GENELAND 4.0.2 (Guillot et al. 2005). We first launched 10 exploratory runs varying the burnin period and the number of iterations to check the convergence of the chains by the end of the MCMC runs. We then performed 20 independent MCMC runs with a burnin period of $2*10^4$ iterations, 10^4 subsequent iterations, correlated frequencies model (F-model), spatial coordinates with uncertainty of approximately 50 m, 400 pixels along the *x* axis and 250 along the *y* axis, and allowing K to vary from 1 to 22.

Results

The complete data set contained 8.17% missing data. Negative controls showed no evidence of sample cross-contamination, and positive controls confirmed that no shifts occurred among different runs of capillary electrophoresis for each SSR duplex.

To verify that sampling within patches included separate genotypes, clonality was previously assessed. Genetic distances between individuals were calculated across all 9 SSR markers (Goldstein et al. 1995) and genotypes between any possible combination of pair of individuals were compared. Out of 295 individuals, only 2 resulted to be clones and belonged to the same population, and even when missing data were taken into account, only 5 pairs of individuals were counted as potential clones. Thus, clonality at 1–3 m sampling distances was considered to be rare in *A. thalictrifolia*.

Evidence of LD among loci was dependent on the correction method employed in the analyses. When FDR was applied, 2 *A. thalictrifolia* sites (i.e., AM3, VE4) each showed evidence of LD at 1 pair of loci (50–21/25.6–16 and 11–3/10–15, respectively) and 1 site (PU2) at 2 pairs of loci (11–3/1–40 and 200.2–4/25.3–33). When Bonferroni was applied, 1 site (PU2) was in LD for the pair 25.3–33/10–15, and 4 sites (i.e., VE1, VE3, PU1, MI1) showed evidence of LD for the pair 25.3–33/11–3. In particular, the latter finding is plausible, given the physical distance of these loci calculated by mapping the respective sequences on the *A. coerulea* Goldsmith genome (~100Kb on scaffold 2; http://www.phytozome.net). Three of 99 probability-tests showed significant departures from HWE proportions following Bonferroni correction: 50–21 in AM2, 7–27.2 in VE3 and 50–7 in AM3. Four of 99 tests for heterozygote deficit were statistically significant: 50–21 in AM2 and VE4, 50–7 in VE3, 11–3 in PU1. Tests for heterozygote excess showed no evidence of departure from HWE expectations. Frequencies (*P*) of null alleles ($0.16 < P < 0.26$) were estimated to be

moderate in 4 loci (i.e., 50–21, 50–7, 25.3–33, 11–3) and 3 populations (i.e., PU1, VE4, and AM1) according to values proposed by Howes et al. (2006), whereas the remaining data set revealed rare null allele frequencies for all loci within all sites. Departure from HWE in the data set affected only a few sites, and the mean population frequency of null alleles per locus was rare. Because both evidence of LD and departure from HWE affect a minor portion of the data set, it was considered unlikely to bias significantly our estimations; thus the entire set of loci was retained for downstream analyses.

Genetic Diversity Measures

All 9 microsatellites were polymorphic across all sites with the exception of 10–15, which was monomorphic in site MI1. Overall, loci contained 8 alleles per locus on average, with a maximum of 22 alleles in locus 7–27.2 in site VE1. Higher levels of allelic richness were found on average within sites located in Valvestino (VE; mean Ar = 7.33), followed by AM (mean Ar = 7.26) and the remaining valleys (PU, mean Ar = 4.45; MI, mean Ar = 3.33). Among all sites, VE5 and MI1 showed the highest and lowest value, respectively; private allelic richness was particularly elevated in sites PU1 and VE5, whereas lowest in VE3. Overall expected heterozygosity across loci and sites was found to be high (mean Hexp = 0.68 ± 0.19 SD (standard deviation); see Table 1).

Population Structure

The AMOVA results (Table 2) revealed 8.42%, 14.38%, and 77.20% of variation among drainages, sites, and individuals, respectively. Both F_{ST} and G_{ST} averaged among sites showed high values (F_{ST} = 0.21, P = 0.000; G_{ST} = 0.20, P = 0.001, 95% CI = 0.1988–0.2044). Overall D_{est} was at least 3 times higher (D_{est} = 0.61, P = 0.001, 95% CI = 0.5984–0.6300). Overall values of Hexp, G_{ST} and D_{est} per locus are available in Table B (see Supplementary Material online).

A square matrix comparing pairwise G_{ST} and D_{est} values is shown in Table 3. G_{ST} between pairs of sampling locations ranged from 0.03 (AM1/AM2) to 0.23 (PU2/AM1), whereas D_{est} values ranged from 0.28 (AM1/AM2) to 0.81 (VE1/PU2). Pairwise D_{est} estimates were found to be consistently at least 3 times higher than corresponding pairwise G_{ST}.

Results of Mantel tests estimating migration-drift equilibrium are summarized in Table C (see Supplementary Material online). A significant pattern of Isolation by Distance (IBD) between genetic and geographic distances was found among all sampling sites, both for linearized G_{ST} and D_{est} (Figure 2A–B). Nevertheless, no significant positive association was found between the degree of scatter and the geographic distance for either G_{ST} or D_{est}. Also, no significant positive correlation was found within the cluster VE as identified by STRUCTURE (Table C of Supplementary Material online). Because the statistical power of these analyses within the 2 remaining clusters (AM and PU–MI) was limited by the low number of sites, IBD could not be thoroughly verified in these cases. The GLS tests on linearized D_{est} and G_{ST} matrixes selected the M0 model assuming homogeneity of variance as the most likely model (D_{est}: lnL = 109.1435 vs 111.9310; G_{ST}: lnL = −157.3586 vs −140.6060). This further confirms that genetic variance does not vary with geographic distance in these cases. Overall, results from Mantel tests and GLS do not support gene flow-drift equilibrium, either on a global or local scale.

The CMDS separated clusters that differed qualitatively between G_{ST} and D_{est} data sets. The CMDS plot built on the G_{ST} matrix (Figure 3A) grouped together the sites located in VE and AM, while placing the sites of PU opposite on the first axis (32.96% of total variance). The second axis (17.67%) separated population MI1. Alternatively, the plot of D_{est} values (Figure 3B) clustered AM and MI, and slightly separated the sites of VE, with VE3 placed between the two groupings on the first axis (46.91% of the total variance). Finally, the second axis (28.78%) separated PU1 and PU2 from the remainder of the sites.

A strong but nonsignificant correlation was recovered by the Mantel test between G_{ST} and D_{est} (r = 0.76, P > 0.05). The AIC selected the nonlinear equation as the best model to fit the data (lnL = −131.8796 vs −41.84355), and consistent results were obtained in the Anova analyses (F = 229.5, degrees of freedom = 1, P < 0.001). A plot of the relationship between D_{est} and G_{ST} is shown in Figure 4.

Nonspatial STRUCTURE analyses indicated 3 possible most likely values of K (Evanno et al. 2005) in the following order of importance: 19, 3, and 11. The subdivision in 19 groups has little biological meaning, as the choice for the best K should ideally aim for the smallest value of K capturing the major structure in the data (Pritchard et al. 2000). Moreover, only 5 of the retrieved clusters could be considered as well defined ($P \geq 0.7$). Hence, we excluded K = 19 from further interpretations. K = 3 resulted in 3 clusters largely

Table 2 Partitioning of molecular variance within *A. thalictrifolia;* global index of fixation across loci based on F_{ST} (Weir and Cockerham 1984) and G_{ST} (Nei 1973); global estimate of population differentiation based on D_{est} (Jost 2008)

Component of variation	SS[a]	Variance component	% Variation	F_{ST}	G_{ST}	D_{est}
Among drainages	176.00	0.33	8.42			
Among populations	245.64	0.57	14.38	0.21*	0.20* (0.1988–0.2044)	0.61* (0.5984–0.6300)
Within populations	1604.33	3.05	77.20			

P values and 95% confidence interval (CI) within brackets (G_{ST} and D_{est}) are based on 1000 permutations.

[a]Sum of squares.

*P < 0.01.

Table 3 Pairwise G_{ST} (above diagonal) and Jost's D_{est} (below) values estimated for every pair of sampled locations ($P < 0.05$ over 1000 bootstrapped matrices after Bonferroni correction; Mantel test between the two triangular matrices: $r = 0.76$, $P > 0.05$)

	VE1	VE2	VE3	VE4	VE5	AM1	AM2	AM3	PU1	PU2	MI1
VE1	—	0.07	0.14	0.07	0.05	0.11	0.09	0.12	0.18	0.21	0.18
VE2	0.55	—	0.10	0.05	0.03	0.08	0.07	0.08	0.15	0.16	0.14
VE3	0.69	0.59	—	0.11	0.08	0.10	0.10	0.13	0.16	0.17	0.17
VE4	0.60	0.38	0.62	—	0.03	0.09	0.10	0.10	0.17	0.18	0.15
VE5	0.55	0.38	0.57	0.26	—	0.07	0.06	0.07	0.13	0.14	0.13
AM1	0.66	0.61	0.61	0.60	0.57	—	0.03	0.05	0.12	0.15	0.13
AM2	0.61	0.60	0.59	0.65	0.56	0.28	—	0.05	0.11	0.15	0.11
AM3	0.75	0.55	0.62	0.58	0.50	0.31	0.37	—	0.08	0.12	0.10
PU1	0.80	0.67	0.65	0.69	0.70	0.58	0.57	0.44	—	0.12	0.18
PU2	0.81	0.69	0.64	0.68	0.63	0.61	0.62	0.53	0.36	—	0.23
MI1	0.76	0.67	0.62	0.68	0.66	0.63	0.62	0.48	0.60	0.69	—

Collection site acronyms refer to Table 1.

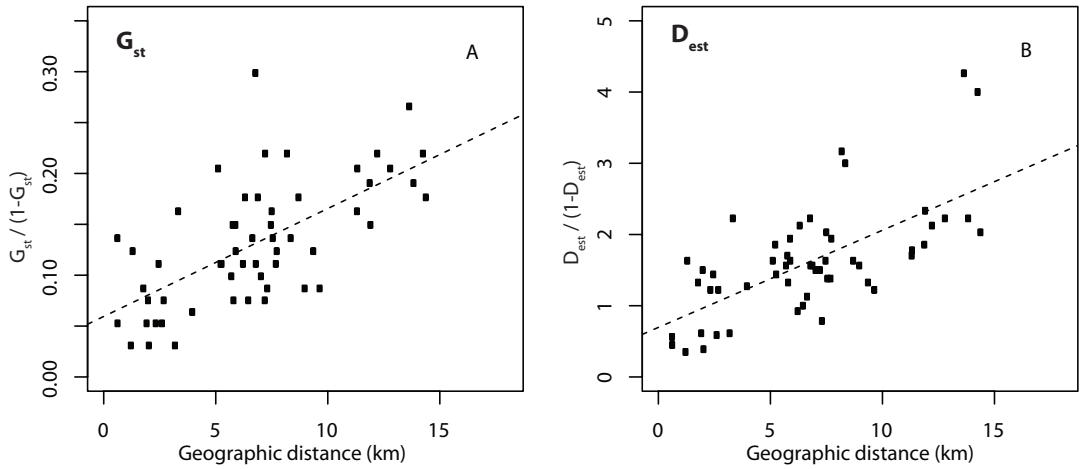

Figure 2. Relationships between genetic distances and straight-line geographic distances among collection sites. (**A, B**) Scatter plot of linearized G_{ST} and D_{est} values versus geographic distances for the complete data set. The dotted line indicates the regression line.

corresponding to drainages where the endemic is distributed (Figure 5A). The first group included 4 locations belonging to the geographic region VE (VE1, VE2, VE4, and VE5). In contrast, VE3 proved to be highly admixed among the 3 clusters. The second cluster included the 3 sites located in AM. The third cluster included the two sites in PU as well as MI, with the latter showing a moderate admixture with AM. With regards to K = 11, the clustering results predominantly distinguished every site (Figure 5B), with the exception of VE5 and VE4, which could not be unambiguously assigned to 1 cluster, and PU1 and PU2, which were identified as a single population.

Spatially explicit GENELAND analyses assuming an F-model distinguished an optimal structure with 11 clusters corresponding to the 11 sites in all independent runs. The mean posterior probability (PP) of simulated parameters along MCMC simulation for each of the 20 runs was calculated, and runs were sorted by decreasing average posterior density following Corander et al. (2003). In the best run, all sites appeared clearly distinguished, with a membership coefficient of PP = 1.

Discussion

Genetic Diversity of *A. thalictrifolia*

Our results show overall high levels of heterozygosity in *A. thalictrifolia*, both as inferred within sites (Table 1) and as an average value for the species (mean Hexp = 0.68 ± 0.19 SD). These results are at odds with available data inferred from other narrow endemics of European distribution, such as the representatives from Sardinia (mean Nei's gene diversity ± SD = 0.035 ± 0.13; Garrido et al. 2012) and the Iberian *A. paui* (mean Hexp ± SD = 0.006 ± 0.01; Martinell et al. 2010), and appear unexpected when considered in light of the biology of the species. The small census and number of recorded populations, the ongoing reduction of population sizes due to regression of suitable habitat (Bonomi et al.

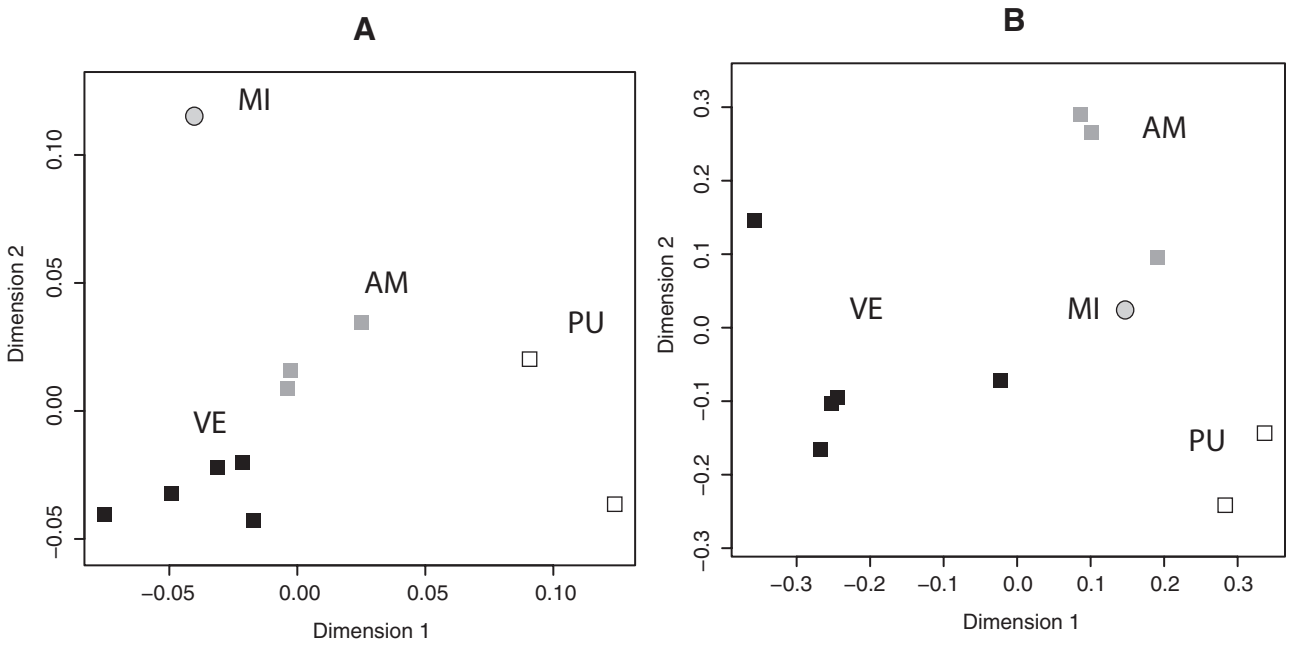

Figure 3. CMDS plot visualizing population structure based on pairwise G_{ST} and D_{est} estimates. Collection sites and identifications of the valleys refer to Table 1 and Figure 1. (**A**) CMDS plot built on pairwise G_{ST} values. (**B**) CMDS plot based on pairwise D_{est} values.

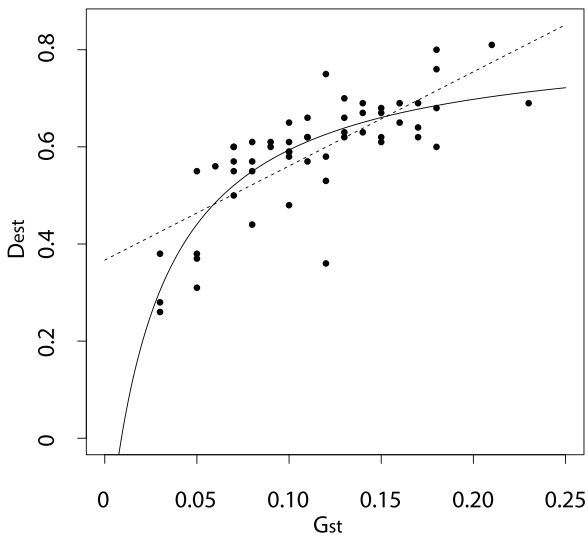

Figure 4. Scatter plot of pairwise D_{est} versus pairwise G_{ST} estimates. The dotted line indicates the interpolation of a linear model as described by $D_{est} = a*G_{ST}$; the solid line indicates the interpolation of a nonlinear model as described by $D_{est} = a*G_{ST}/(1+b*G_{ST})$.

2008), and the retrieved low levels of gene flow, are expected to strengthen the effect of demographic, environmental, and genetic stochasticity, which in turn decrease genetic diversity (Frankham et al. 2002). On the other hand, the strong inbreeding depression that has been found in *Aquilegia* species is likely to counteract genetic homogenization, and in this respect, it could be key to interpret our results. Although no direct evidence is available on *A. thalictrifolia*, studies on North American species (e.g., *A. formosa*, *A. pubescens*, *A. coerulea*, *A. canadensis*; Montalvo [1994]; Herlihy and Eckert [2002]; Brunet et al. [2006]; Yang et al. [2010]) have found inbreeding depression to dramatically limit survival of progeny originating from selfing or biparental crossing, thus greatly favoring outcrossing with nonrelatives and maintenance of high heterozygosity within taxa. In this regard, it is notable that the levels of heterozygosity recovered in *A. thalictrifolia* are comparable to those found in *A. coerulea* (Brunet et al. 2012; mean Hexp = 0.74, SD = 0.02), a taxon characterized by a wide distribution area in Rocky Mountains. Within the European scenario, the low level of heterozygosity recovered in *A. paui* is consistent to the shift of this species to autogamy (Martinell et al. 2010, 2011). As to the Sardinian endemics (Garrido et al. 2012), until empirical data are produced, it remains conjectural to recognize if reduced levels of inbreeding depression found also in these species can account for the low heterozygosity recovered. Other factors that could have a bearing on the interpretation of our results could be both methodological and intrinsic to the evolutionary history of the species. For instance, past herbarium specimens (observed at FI, K, HBBS, PAD, TR) indicate that other populations existed besides those that we could find in the field, and the observed regression in population size of the current populations suggests that our results could be linked to a very recent demographic decline (Bonomi 2008) that is not yet evident in heterozygosity levels. Indeed, allelic diversity decreases faster than heterozygosity

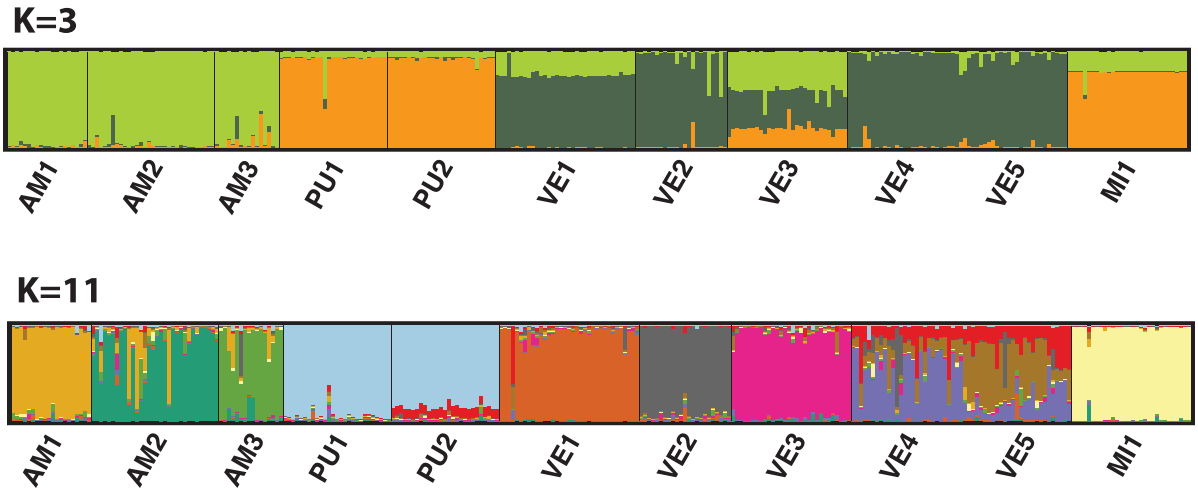

Figure 5. Bar plot showing the assignment of individuals to the clusters inferred by STRUCTURE, and their respective levels of admixture. Each color represents 1 group, and the length of colored bars depicts the fractional assignment of individuals to the genetic clusters. Collection site acronyms refer to Table 1. **(A)** Population structure for K = 3 genetic clusters. (**B**) Population structure for K = 11 genetic clusters.

after a reduction in population size when loci evolve under the IAM model (Maruyama and Fuerst 1985). Additionally, *A. thalictrifolia* is a long-lived plant, with a generation time of approximately 12 years (Bonomi et al. 2008), which is expected to reduce the loss of alleles caused by genetic drift and the incidence of mutations (Young et al. 1996; Honnay and Jacquemyn 2007). High levels of genetic diversity could also be maintained in the populations owing to the presence of a persistent soil seed bank (Honnay et al. 2008), similar to the case described by Mattana et al. (2012) on the endemics of Sardinia. Finally, introgression with other congenerics should be considered, as evidence for hybridization with the geographically close *A. einseleana* emerges from the peripheral populations in Valsugana and Tramonti di Sopra (Figure 1A). However, genetic diversity produced in a parallel work (preliminary data) for these populations using the same set of SSRs as in the present study shows levels of heterozygosity (Valsugana, Hexp. = 0.74; Tramonti di Sopra, Hexp. = 0.88) within the range observed in *A. thalictrifolia*, and the limited gene flow among populations recovered in this study discredits introgression as a major cause of high genetic diversity in the core distribution of the species.

Population Structure of *A. thalictrifolia*

Analyses performed in the present study confirmed the presence of a strong population structure within *A. thalictrifolia*, a narrow endemic with fragmented distribution, and specialized ecological niche in the heterogeneous Alpine landscape.

The genetic variability of *A. thalictrifolia* seems to be organized in two possible structures. First, STRUCTURE analyses, supported by AMOVA, identified K = 3 as the most likely number of clusters, which largely correspond to main drainages that partition the species distribution: that is, VE, AM, and the area including PU and MI. Mantel tests suggest that the limited connectivity among these 4 areas is one of the principal drivers of genetic structuring. In fact, despite a significant association between genetic and geographic distances, the residuals of their linear regression are scattered with respect to geographic straight-line distances, and pairwise genetic distances are high (see below), thus suggesting the predominant role of genetic drift over migration (Hutchinson and Templeton 1999). Along with orographic discontinuities, the forests of Norway spruce and beech that typically surround the rock ledges hosting *A. thalictrifolia* may further reduce long-range pollen dispersal by typical pollinators such as hymenopters, lepidopters, and dipters (Lavergne et al. 2005; Medrano et al. 2006; Bastida et al. 2010; Martinell et al. 2011), which are more effective in dispersing pollen in open land than in closed forests (Kreyer et al. 2004; Kamm et al. 2010). On the other hand, STRUCTURE analyses suggest that a moderate gene flow is maintained especially between geographically close sites. The fact that the species occurs at seeps, places animals are drawn to, suggests that connectivity among populations may be maintained by seed dispersal. We noticed substantial predation of columbine follicles in combination with the presence of ungulate feces in several populations of *A. thalictrifolia* (M Lega, personal observation). Consistently, Garrido et al. (2012) suggested that seed dispersal by herbivore endozoochory (Manzano et al. 2005; Manzano and Malo 2006) could contribute to maintain genetic variability among populations of *Aquilegia* endemics in Sardinia, and Martinell et al. (2011) documented goat predation of fruits in the endemic Iberian *A. paui*.

The second possibility of genetic structuring is represented by the individual collection sites: These were all distinguished by GENELAND and, to a large extent, also by STRUCTURE analyses when results for K = 11 are considered. These clusters were supported by the AMOVA outputs, and consistently, high values of G_{ST} and D_{est} (> 0.15; cf. Leng

and Zhang [2011]) were recovered in estimates both across populations and in single pairwise comparisons. In particular, D_{est} depicted a clear and substantial differentiation among collection sites. Indeed, values from these indexes appear particularly high even when compared with those calculated for *A. coerulea* (F_{ST} = 0.098, D_{est} = 0.35; Brunet et al. 2012). Similar evidence is drawn by comparisons with other SSR studies on herbaceous plants with unspecific pollination syndromes, such as *Campanula tyrosoides* (mean Hexp = 0.75, SD = 0.06; G_{ST} = 0.15, D_{est} = 0.58; Kuss et al. 2011) and *Dryas octopetala* (mean Hexp = 0.61, SD = 0.08; mean pairwise F_{ST} and D_{est} values: 0.25 and 0.52, respectively; Vik et al. 2010). According to Leng and Zhang (2011), 3 hypothetical scenarios could explain the occurrence of elevated values of D_{est} and G_{ST}: strong population differentiation with very weak gene flow (Nm<1); very small population size ($N \leq 100$); and low mutation rate ($\mu \leq 10^{-4}$) coupled with very ancient population differentiation. On the basis of the evidence presented herein, the first scenario seems to apply best to *A. thalictrifolia*. However, cases of populations with an estimated number of flowering individuals lower than 100 were recorded during field work, which suggests that the second scenario could also contribute to our results. With regards to the third scenario, a low mutation rate is not likely because of the dinucleotidic nature of 8 SSRs and the high number of alleles detected on average per locus.

Evolutionary Insights on Population Differentiation Within *A. thalictrifolia*

Further evolutionary insights on the process of population differentiation within *A. thalictrifolia* come from the comparative interpretation of the population structures depicted by G_{ST} and D_{est} indexes (Heller and Siegismund 2009; Meirmans and Hedrick 2011). The different properties of the two measures are reflected by the nonlinear relationship between them: Although D_{est} is more sensitive to population structure than G_{ST} when gene flow and within population diversity are high, G_{ST} performs better in detecting population structure when the effect of drift is higher and within population diversity is low (Jost 2008, 2009; Leng and Zhang 2011; Raeymaekers et al. 2012). A recent work by Raeymaekers et al. (2012) demonstrated that, on short time scales, from late Pleistocene onwards, G_{ST} provides a correct picture of genetic structure as recently shaped by migration and drift, whereas D_{est}, owing to the much slower process necessary to reach equilibrium, is less influenced by contemporary demographic events, and thus better reflects the signature of previous colonization histories.

In our case study, the clustering of sites from AM and MI recovered in the D_{est} CMDS, but not in the G_{ST} CMDS, indicates that a past continuum between the two drainages could have existed, followed by a more recent reduction in gene flow. This hypothesis is compatible with the potential of *A. thalictrifolia* to establish transient populations, as documented in herbarium collections. Moreover, the distinctiveness of populations from VE found by D_{est} suggests some degree of past isolation from the remnant populations. We interpret this result in light of the Pleistocene history that characterizes the peaks of Mount Tombea, a mountain ridge within the VE region whose summits and lateral slopes remained free from the ice cover during glacial periods (Avanzini 1999), thus likely acting as a glacial refugium for calcicolous plants. The role of refugia on calcareous bedrock between the Dolomites and Lake of Como was previously emphasized by Schönswetter et al. (2005). During the last glacial maximum, these formations hosted most of the endemics of the whole Eastern Alps, including an exceptionally high number of taxa in the Tombea mountain chain (Prosser 1999; Tribsch 2004).

Conclusions

The key role of climatic oscillation and geographic isolation in shaping the *Aquilegia* radiation in Europe has been previously proposed in the work by Fior et al. (2013), and the present study adds evidence of the effects of these forces at a narrower geographic scale. These results are complementary to the evidence produced on the Sardinian endemics (Garrido et al. 2012) in supporting allopatry as a major determinant for establishment of intraspecific diversification and speciation patterns. In this study, natural barriers such as topological constraints corresponding to main drainages appear as major natural barriers to genetic homogenization. However, the typical habitat requirement of stenoendemics is a likely complementary force in limiting geographic connectivity among populations, and in fact, its role may be crucial in the maintenance of species boundaries. This was proved true for North American mountainous species *A. formosa* and *A. pubescens* (Chase and Raven 1975), and strong differentiation of taxa is reinforced by their different pollination syndrome (Arnold and Hodges 1994; Fulton and Hodges 1999; Cooper et al. 2010). Although such reinforcement provided by diversification of floral traits appears to be lacking in the European species, adaptation in vegetative traits and shift in reproductive strategy has previously been identified as primary forces of ecological diversification (Alcantara et al. 2010; Castellanos et al. 2011; Martinell et al. 2011). Further work on the loci underlying adaptive traits and their interplay with landscape features will thus be key to elucidate the drivers of speciation processes in European columbines.

Supplementary Material

Supplementary materials can be found at http://www.jhered.oxfordjournals.org/.

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