RESEARCH ARTICLE

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# Comparative epigenetic and genetic spatial structure of the perennial herb *Helleborus foetidus*: Isolation by environment, isolation by distance, and functional trait divergence[1]

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**PREMISE OF THE STUDY:** Epigenetic variation can play a role in local adaptation; thus, there should be associations among epigenetic variation, environmental variation, and functional trait variation across populations. This study examines these relationships in the perennial herb *Helleborus foetidus* (Ranunculaceae).

**METHODS:** Plants from 10 subpopulations were characterized genetically (AFLP, SSR markers), epigenetically (MSAP markers), and phenotypically (20 functional traits). Habitats were characterized using six environmental variables. Isolation-by-distance (IBD) and isolation-by-environment (IBE) patterns of genetic and epigenetic divergence were assessed, as was the comparative explanatory value of geographical and environmental distance as predictors of epigenetic, genetic, and functional differentiation.

**KEY RESULTS:** Subpopulations were differentiated genetically, epigenetically, and phenotypically. Genetic differentiation was best explained by geographical distance, while epigenetic differentiation was best explained by environmental distance. Divergence in functional traits was correlated with environmental and epigenetic distances, but not with geographical and genetic distances.

**CONCLUSIONS:** Results are compatible with the hypothesis that epigenetic IBE and functional divergence reflected responses to environmental variation. Spatial analyses simultaneously considering epigenetic, genetic, phenotypic and environmental information provide a useful tool to evaluate the role of environmental features as drivers of natural epigenetic variation between populations.

**KEY WORDS** epigenetic variation; functional divergence; genetic variation; isolation by distance; isolation by environment; local adaptation; Ranunculaceae; spatial structure

Characterizing spatial patterns of genetic diversity in natural populations and disentangling their causal processes are central to understanding the ecology and evolution of natural populations (Wright, 1943; Silvertown and Antonovics, 2001; Epperson, 2003). These investigations have frequently documented patterns conforming to expectations from the Wright (1943) isolation by distance (IBD hereafter) model, in which genetic differentiation increases with geographical distance between populations (Meirmans, 2012; Sexton et al., 2014). Under Wright's IBD formulation, genetic differentiation between spatially separated populations is the consequence of restricted gene dispersal, which leads to genetic drift having a greater impact than gene flow between populations (Rousset, 1997; Epperson, 2003). Originally, IBD models did not take into consideration the possible modifications of gene flow arising from habitat discontinuities or environmental features at the landscape or regional scales. Recently, increasing recognition that the interactions between organisms and features of the environment can shape spatial patterns of gene flow and genetic variation (Foll and Gaggiotti, 2006; Lee and Mitchell-Olds, 2011) has given rise to the concept of isolation by environment (IBE hereafter), in which genetic and environmental distances are positively correlated independently of geographic distance (Orsini et al., 2013). A recent review points to the pervasiveness of genetic IBE in natural populations (Sexton et al., 2014).

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In contrast with the abundant information on the spatial structure of genetic diversity, spatial patterns of epigenetic variation and their causal processes remain largely unexplored (Burggren, 2016; Herrera et al., 2016; Whipple and Holeski, 2016). Particularly scarce are data documenting epigenetic IBD and IBE comparable to those known for genetic variation. Limited evidence suggests that epigenetic divergence tends to increase with the spatial scale of studies (Herrera et al., 2016), which is reminiscent of ordinary genetic IBE. Evidence of epigenetic IBD was provided by two recent studies revealing small-scale, individual-level spatial structuring of epigenetic diversity (Herrera and Bazaga, 2016; Herrera et al., 2016). Negative linear relationships were found between the kinship coefficient for pairs of individuals computed from epigenetic markers and their spatial separation, but a formal extrapolation from individual- to population-level IBD was not attempted. These studies advanced the hypothesis that spatial epigenetic structure can conform to IBE, even when genetic variation does not, if environmental differences between populations are more important than geographical distances as predictors of epigenetic differentiation (Herrera and Bazaga, 2016; Herrera et al., 2016). To our knowledge, however, the possibility that epigenetic variation between populations conforms to IBE has been not explicitly tested (but see Schulz et al., 2014; Huang et al., 2015). Since local adaptation is accepted as a major process causing IBE (Orsini et al., 2013; Wang and Bradburd, 2014), the current dearth of information on epigenetic IBE is hindering progress in understanding the possible adaptive value of epigenetic responses to environmental variation.

We present in this paper an analysis of the spatial structure of epigenetic and genetic variation across populations of the perennial herb *Helleborus foetidus*, in combination with data on habitat characteristics and functional traits that are potentially subject to selection. By focusing on between-population spatial structure, incorporating information on environmental features, and simultaneously testing for IBD and IBE of epigenetic and genetic variation, results of this study extend those of Herrera et al. (2016) for the same species, which dealt exclusively with individual-level IBD and did not take environmental features of habitats into account. First, we tested whether individual-level, small-scale epigenetic and genetic IBD scale up to produce epigenetic and genetic population-level, landscape-scale IBD. Second, we tested the prediction that epigenetic variation between populations should predominantly conform to IBE even if genetic variation predominantly conforms to IBD (Herrera and Bazaga, 2016; Herrera et al., 2016). Such spatially decoupled patterns of genetic and epigenetic divergence across populations would provide circumstantial evidence in support of environmentally driven epigenetic differentiation between populations. Third, we tested the relative explanatory value of geographical and environmental distance between conspecific populations as predictors of genetic and epigenetic differentiation. Results from these three tests will allow us to address the broader question of the adaptive value of epigenetic divergence between populations, an aspect that has been insufficiently investigated (but see Herrera and Bazaga, 2010; Dubin et al., 2015; Foust et al., 2016; Keller et al., 2016). Last, by analogy with genetic IBE caused by local adaptation (Orsini et al., 2013; Wang and Bradburd, 2014), we tested whether population divergence in functional, fitness-related phenotypic traits could be parsimoniously accounted for by the combined effects of environmental and epigenetic differences.

## MATERIALS AND METHODS

***Study plant and field sampling***—*Helleborus foetidus* L. (Ranunculaceae) is a perennial herb widely distributed in western and southwestern Europe. It occurs in contrasting habitats ranging from open scrub to conifer and broad-leaved forests from sea level to 2100 m a.s.l. (Mathew, 1989). Flowers are hermaphroditic, self-compatible, and nearly exclusively pollinated by bumble bees. Dispersed seeds either remain under the parent plant or are moved short distances by ants, and seedling recruitment mostly occurs within ~2 m of maternal parents (Herrera et al., 2002). Sampling for this study was conducted at 10 locations in the Sierra de Cazorla, southeastern Spain (see map of sampling locations in appendix S1 of Medrano et al., 2014). Distances between sites ranged between 0.7–19.1 km. At each site ("subpopulations" hereafter), 20 widely spaced, inflorescence-bearing plants were selected. Plants were the same ones studied by Medrano et al. (2014) and Herrera et al. (2016). Young leaves were collected from each plant, placed in paper envelopes and dried at ambient temperature in containers with silica gel for subsequent DNA extraction. In addition, all plants were characterized phenotypically by the following 20 life history, fecundity, and leaf functional traits, all of which may directly or indirectly affect the fitness of individuals and therefore be subject to selection (Medrano et al., 2014; García-Cervigón et al., 2016): number of vegetative, reproductive, and total ramets; age of flowering ramets; basal diameter of inflorescence; length of floral perianth; number of follicles per flower; number of flowers produced; number of follicles and seeds ripened; seed mass; leaf carbon isotope ratio; specific leaf area; leaflet length, width, area, and mass; stomatal length and density; and stomatal index. Methods were described in detail by Medrano et al. (2014), and individual trait values are accessible in the Dryad Data Repository (doi:10.5061/dryad.fr2k8).

Six environmental features were used to characterize sampling sites ecologically: elevation (range 735–1805 m a.s.l.), life zone (meso-, supra- or oro-mediterranean, following the classification of Gómez Mercado, 2011), successional status (undisturbed plant community vs. signs of natural or anthropogenic disturbance), groundcover layer (predominantly continuous herbaceous layer vs. prevailing bare ground), shrub layer (present vs. absent), and tree layer (dense forest with closed canopy vs. open woodland with sparse trees). These coarse environmental variables were sufficient to depict the main ecological differences between sites (Table 1) and are reasonable proxies for unmeasured environmental variables that are known to influence fecundity, demography, seed dispersal, seedling emergence, early growth, and survival in *H. foetidus* (soil variables, water stress, light regime, disturbance, vegetation type, and tree, shrub, and herb cover; Manzaneda et al., 2005; Rey et al., 2006a, b; Garrido et al., 2007; Rey and Manzaneda, 2007; Herrera et al., 2014; García-Cervigón et al., 2015, 2016).

***Genetic and epigenetic fingerprinting***—All plants sampled were characterized genetically and epigenetically. Total genomic DNA was extracted from dry leaf samples using a Qiagen DNeasy Plant Mini Kit and the manufacturer's protocol. Genetic fingerprints for each plant were obtained using amplified fragment length polymorphism markers (AFLP) and nuclear microsatellites (SSR hereafter). The AFLP analyses were performed using standard protocols involving the use of fluorescent-dye-labeled selective primers and a total of eight PstI + 2 / MseI + 3 primer pairs. The SSR genotyping

**TABLE 1.** Location and ecological characteristics of the 10 sites where *Helleborus foetidus* plants were sampled. Longitude, latitude, and elevation values are means for individual plants at each site. Life zones refer to the meso-, supra-, and oro-mediterranean vegetational belts.

| Code | Site | Longitude (° W) | Latitude (° N) | Elevation (m a.s.l.) | Life zone | Successional status | Tree layer | Shrub layer | Ground layer |
|---|---|---|---|---|---|---|---|---|---|
| CAN | El Cantalar | 2.90251 | 37.97227 | 774 | Meso | Disturbed | Dense | Absent | Bare ground |
| CFU | Cañada de las Fuentes | 2.97597 | 37.84098 | 1352 | Supra | Disturbed | Dense | Present | Bare ground |
| ESP | La Espinarea | 2.95858 | 37.87462 | 1143 | Supra | Disturbed | Sparse | Absent | Herbaceous |
| FBE | Fuente Bermejo | 2.84049 | 37.92730 | 1529 | Supra | Undisturbed | Sparse | Absent | Herbaceous |
| MES | Arroyo de la Mesa | 2.92849 | 37.90274 | 1028 | Supra | Disturbed | Sparse | Absent | Herbaceous |
| NAV | Las Navillas | 2.90972 | 37.93593 | 1241 | Supra | Undisturbed | Dense | Absent | Bare ground |
| PLL | Puerto Llano | 2.95929 | 37.81011 | 1801 | Oro | Undisturbed | Sparse | Present | Herbaceous |
| SCA | Cuesta de la Víbora | 2.96106 | 37.83961 | 1546 | Supra | Undisturbed | Dense | Present | Bare ground |
| TEJ | Tejerina | 2.90707 | 37.97731 | 737 | Meso | Disturbed | Dense | Present | Bare ground |
| VCU | Valdecuevas | 2.86859 | 37.91198 | 1401 | Supra | Undisturbed | Dense | Absent | Herbaceous |

was based on 11 polymorphic loci (MERPD Consortium et al., 2013). Individual AFLP ($N$ = 270 loci) and SSR fingerprints used here are those of Medrano et al. (2014) and Herrera et al. (2016), respectively.

The methylation-sensitive amplified polymorphism technique (MSAP; Schulz et al., 2013; Guevara et al., 2017) was used to characterize plants epigenetically. Genome-wide analyses of DNA methylation in nonmodel organisms without a reference genome are still challenging. In ecological epigenetic investigations, when detailed information on the genomic location of epigenetic markers is not essential (Rausher and Delph, 2015), anonymous MSAP markers can be validly applied (Preite et al., 2015; Foust et al., 2016; Wilschut et al., 2016) despite their acknowledged limitations (Schrey et al., 2013; Fulneček and Kovařik, 2014). The MSAP technique is a modification of the standard AFLP method that uses the methylation-sensitive restriction enzymes HpaII and MspI in parallel runs in combination with another restriction enzyme. Differences in the products obtained with HpaII and MspI reflect different methylation states at the cytosines of anonymous CCGG sites recognized by HpaII or MspI (see Schulz et al., 2013; Alonso et al., 2016; for further details). MSAP assays were conducted using four HpaII-MspI + 2 / MseI + 3 primer combinations. The "mixed scoring 1" transformation scheme of Schulz et al. (2013) was applied to the presence–absence matrices for MSAP fragments obtained with the four HpaII–MseI and MspI–MseI primer combination pairs. Under this scheme, MSAP fragments are transformed into two distinct sets of MSAP markers, corresponding to unmethylated and methylated types ($u$ and $M$ markers, respectively; Schulz et al., 2013). Plants sampled were characterized epigenetically by presence–absence scores for $u$- and $M$-type MSAP markers ($N$ = 105 and 142, respectively). Individual MSAP fingerprints used here are those of Herrera et al. (2016).

***Data analysis*—**Genetic and epigenetic IBD and IBE models predict direct relationships between pairwise population differentiation and geographical and environmental distance, respectively (Rousset, 1997; Epperson, 2003; Sexton et al., 2014; Wang and Bradburd, 2014; Herrera et al., 2016). Parameters describing the spatial structure for a given genetic or epigenetic data set, however, may depend on the type of marker chosen (Hardy et al., 2006; Jump and Peñuelas, 2007; Herrera and Bazaga, 2016). Two sets each of genetic (SSR, AFLP) and epigenetic (MSAP $M$- and $u$-type) markers were used to assess the spatial structure of genetic and epigenetic variation across *H. foetidus* subpopulations. Using dominant markers to test spatial structure models requires the application of allele frequency-based approaches (sensu Bonin et al., 2007), which in turn necessitates information on the inbreeding coefficient of sampled individuals (Hardy, 2003; Ley and Hardy, 2013). The inbreeding coefficient for plants in our sample was estimated from the codominant SSR data, and the figure obtained ($F_{IS}$ = 0.141) was then used in every analysis that required computations of allele frequencies from dominant markers (AFLP and MSAP).

All statistical analyses were carried out using the R environment (R Core Team, 2014). The adegenet package (Jombart, 2008) was used to compute overall and pairwise differentiation ($F_{ST}$) between subpopulations based on codominant SSR data, while the corresponding $F_{ST}$ estimates based on dominant AFLP and MSAP markers were computed with the program AFLP-SURV (Vekemans et al., 2002). Statistical significance of overall $F_{ST}$ estimates (i.e., departure from $H_0$: $F_{ST}$ = 0) was in all cases tested using permutations. Pairwise $F_{ST}$ matrices for genetic and epigenetic markers are presented in Appendix S1 (see Supplemental Data with this article). Genetic and epigenetic differentiation between subpopulations was estimated with $F_{ST}/(1 - F_{ST})$ (Rousset, 1997).

Matrices of pairwise geographical, environmental, and phenotypic distances between subpopulations were computed (Appendix S1). Geographical distances were log-transformed for all analyses (Rousset, 1997). Environmental distances were obtained with the generalized Gower dissimilarity index (range = 0–1) implemented in the daisy function of package cluster (Maechler et al., 2016). The statistically nonsignificant correlation between geographical and environmental distance matrices (Mantel coefficient = 0.212, $P$ = 0.09) suggested a weak relationship between geographical separation and ecological disparity between locations. Phenotypic distances between subpopulations were calculated by first obtaining subpopulation mean trait values and standardizing them (mean = 0, standard deviation = 1), and then applying to these data the daisy function with euclidean metric. Geographical, environmental and phenotypic distance distributions did not depart significantly from normality ($P$ = 0.11, 0.73, and 0.68, respectively; Shapiro–Wilk normality tests).

Following Sexton et al. (2014), a two-step procedure was adopted to test for IBD and IBE. In a first step, simple models were evaluated for each class of genetic and epigenetic markers to test whether genetic or epigenetic differentiation between subpopulations was directly related to geographical (Rousset, 1997; Guillot et al., 2009) and environmental distance matrices (Orsini et al., 2013; Sexton et al., 2014; Wang and Bradburd, 2014). Simple Mantel tests were used in these analyses (Diniz-Filho et al., 2013). In a second step, we tested for direct relationships between genetic and epigenetic differentiation and geographical distance after controlling for the effect of environmental distance, and for direct relationships

between genetic and epigenetic differentiation and environmental distance after controlling for the effect of geographical distance (Sexton et al., 2014). Partial Mantel tests were used in these analyses (Diniz-Filho et al., 2013). Simple and partial Mantel tests were conducted using the function mantel in the package ecodist (Goslee and Urban, 2007), and statistical significance was obtained by permutation. Simple and partial Mantel tests are suitable to test hypotheses that specifically concern dissimilarities (Legendre and Fortin, 2010; Legendre et al., 2015), such as IBD and IBE hypotheses tested here, but they have been criticized for having inflated type I error rate and low statistical power, and the controversy on their validity in hypothesis testing remains unresolved (Diniz-Filho et al., 2013; Guillot and Rousset, 2013; Legendre et al., 2015). As an alternative to Mantel procedures, we also used Wang's (2013) method based on multiple matrix regression with randomization (MMRR). Each genetic and epigenetic differentiation matrix was regressed simultaneously against geographical (log transformed) and environmental distance matrices. This method simultaneously assessed the effects of geographic distance and environmental distance on genetic or epigenetic differentiation matrices, rather than evaluating correlations after the effect of geography or environment have been removed, as in the partial Mantel test. Computations were done using the MMRR function for R of Wang (2013) available from the Dryad Data Repository (doi:10.5061/dryad.kt71r). Before the analyses, every dissimilarity matrix was scaled and centered (mean = 0, standard deviation = 1) to obtain comparable standardized linear regression coefficients.

To dissect the relative importance of geographical and environmental distances as predictors of genetic and epigenetic divergence between subpopulations, multiple linear regressions were run when genetic or epigenetic pairwise differentiation between subpopulations was the response variable, and the corresponding geographical distance (log transformed) and environmental distance were the linear predictors. $R^2$ of regressions was then decomposed into additive components estimating the "relative importance" of each predictor (Grömping, 2015) using the LMG method described by Grömping (2007) and implemented using the function calc.relimp in the package relaimpo (Grömping, 2006). To facilitate comparisons, predictor importances were expressed as proportions of total variance explained. Standardized regression coefficients from MMRR analyses provided additional information on the relative importance of geographic and environmental distances as predictors of epigenetic and genetic differentiation.

Phenotypic divergence between populations was tested using multivariate and univariate analyses of variance on functional traits. Predicted relationships under epigenetic IBE by adaptation were tested by running MMRR with phenotypic distance as the dependent matrix and geographical, environmental, genetic (AFLP data only) and epigenetic (*u*-type markers only) distances as independent (predictor) matrices.

## RESULTS

***Genetic, epigenetic, and phenotypic differentiation of subpopulations***—Subpopulations were differentiated genetically and epigenetically, as shown by overall $F_{ST}$ values being significantly greater than zero for all marker types (Table 2). Genetic differentiation estimates obtained with AFLP and SSR markers were closely similar, and substantially lower than estimates of epigenetic differentiation obtained with MSAP *M*- and *u*-type markers (Table 2). Genetic and epigenetic differentiation values were largely independent of each other, as shown by statistically nonsignificant correlations between $F_{ST}$ matrices for AFLP and SSR markers, on one side, and those for MSAP *M*-type (Mantel coefficient = 0.287 and 0.347, $P = 0.12$ and 0.08, for AFLP and SSR, respectively) and *u*-type markers, on the other (Mantel coefficient = 0.185 and 0.212, $P = 0.24$ and 0.23, for AFLP and SSR, respectively).

**TABLE 2.** Overall genetic (AFLP and SSR) and epigenetic (MSAP) differentiation between *Helleborus foetidus* subpopulations.

| Marker type | Marker | $F_{ST}$ | *P*-value |
|---|---|---|---|
| Genetic | AFLP | 0.0442 | <0.0001 |
| | SSR | 0.0723 | <0.0001 |
| Epigenetic | MSAP *M*-type | 0.1480 | <0.0001 |
| | MSAP *u*-type | 0.1707 | <0.0001 |

*Notes:* The null hypothesis $H_0$: $F_{ST} = 0$ was tested, and *P* values were obtained using permutation tests (individuals randomly permuted across populations) with $N = 10^4$ repetitions.

Subpopulations were phenotypically heterogeneous, as denoted by highly significant multivariate analysis of variance (Wilk's lambda = 0.00268, df = 9, 133, $P << 0.0001$). Univariate analyses of variance showed that all functional traits differed significantly among subpopulations (online Appendix S2).

***Isolation by distance***—Simple Mantel tests revealed that both genetic and epigenetic differentiation between subpopulations conformed to IBD. Regardless of marker type, there were positive linear, statistically significant (or marginally significant, MSAP *M*-type) relationships between pairwise matrices of subpopulation differentiation $[F_{ST}/(1 - F_{ST})]$ and (log) geographical distance ("Simple test" columns in Table 3). Genetic and epigenetic differentiation between subpopulations differed in two important respects. First, geographical distance was a better predictor of genetic than of epigenetic differentiation, as denoted by larger Mantel statistics for AFLP and SSR markers relative to MSAP markers (Table 3). And second, over the range of geographical distances studied, mean predicted epigenetic differentiation between subpopulations for a given distance was consistently greater than mean predicted genetic differentiation (Fig. 1).

Partial Mantel tests revealed that, after accounting for environmental distance between subpopulations, genetic differentiation remained significantly related to geographical distance (Table 3). In contrast, the relationship between epigenetic differentiation and geographical distance was considerably weakened after partialling on environmental distance as denoted by substantial reductions of Mantel coefficients, which either failed to reach statistical significance (MSAP *M*-type) or barely met the significance threshold (MSAP *u*-type) (Table 3). Results of MMRR analyses corroborated partial Mantel tests, revealing strong IBD for genetic variation regardless of marker type, and nonsignificant (MSAP *M*-type) or barely significant (MSAP *u*-type) IBD for epigenetic variation (Table 4).

***Isolation by environment***—Simple Mantel tests confirmed expectations from IBE for both genetic and epigenetic variation, as denoted by statistically significant (marginally in the case of MSAP *M*-type) relationships between genetic and epigenetic differentiation and environmental distance (Table 3). Fitted differentiation–environmental distance regressions indicated that, irrespective of

**TABLE 3.** Summary of Mantel tests relating genetic (AFLP and SSR markers) and epigenetic (MSAP *M*-type and *u*-type markers) differentiation matrices [$F_{ST}/(1 - F_{ST})$] between subpopulations of *Helleborus foetidus* with matrices of geographical and environmental distance. Significance of matrix correlations was tested by permutations with $10^4$ repetitions.

| | Geographical distance matrix | | | | Environmental distance matrix | | | |
|---|---|---|---|---|---|---|---|---|
| | Simple test | | Partialled on environmental distance | | Simple test | | Partialled on geographical distance | |
| Differentiation matrix | Mantel coefficient | *P* | Mantel coefficient | *P* | Mantel coefficient | *P* | Mantel coefficient | *P*- |
| Genetic | | | | | | | | |
| AFLP | 0.500 | 0.0005 | 0.419 | 0.0042 | 0.431 | 0.0035 | 0.349 | 0.014 |
| SSR | 0.416 | 0.0002 | 0.334 | 0.0038 | 0.370 | 0.012 | 0.275 | 0.053 |
| Epigenetic | | | | | | | | |
| MSAP *M*-type | 0.174 | 0.083 | 0.093 | 0.36 | 0.268 | 0.057 | 0.212 | 0.11 |
| MSAP *u*-type | 0.286 | 0.0027 | 0.176 | 0.041 | 0.400 | 0.0021 | 0.336 | 0.0051 |

marker type, for a given environmental distance between subpopulations the mean predicted epigenetic differentiation tended to exceed mean genetic differentiation, particularly at greater environmental distances (Fig. 1).

Partial Mantel tests showed that, after accounting for geographical distance, the strength of the relationship between genetic and environmental distance was weakened, as denoted by reduced Mantel coefficients (Table 3). In contrast, after partialling on geographical distance, the relationship between epigenetic and environmental distance remained highly significant in the case of MSAP *u*-type markers (Table 3). The relationship for MSAP *M*-type markers remained statistically nonsignificant. MMRR analyses confirmed the preceding patterns (Table 4).

***Explanatory value of geographical vs. environmental distance***—Relative importance analyses revealed a sharp contrast between genetic and epigenetic differentiation with regard to the comparative explanatory value of geographical and environmental distance (Fig. 2). Geographical distance was by far the most important predictor of genetic differentiation between *H. foetidus* subpopulations irrespective of marker type. In contrast, geographical and environmental distance had roughly similar importance as predictors of

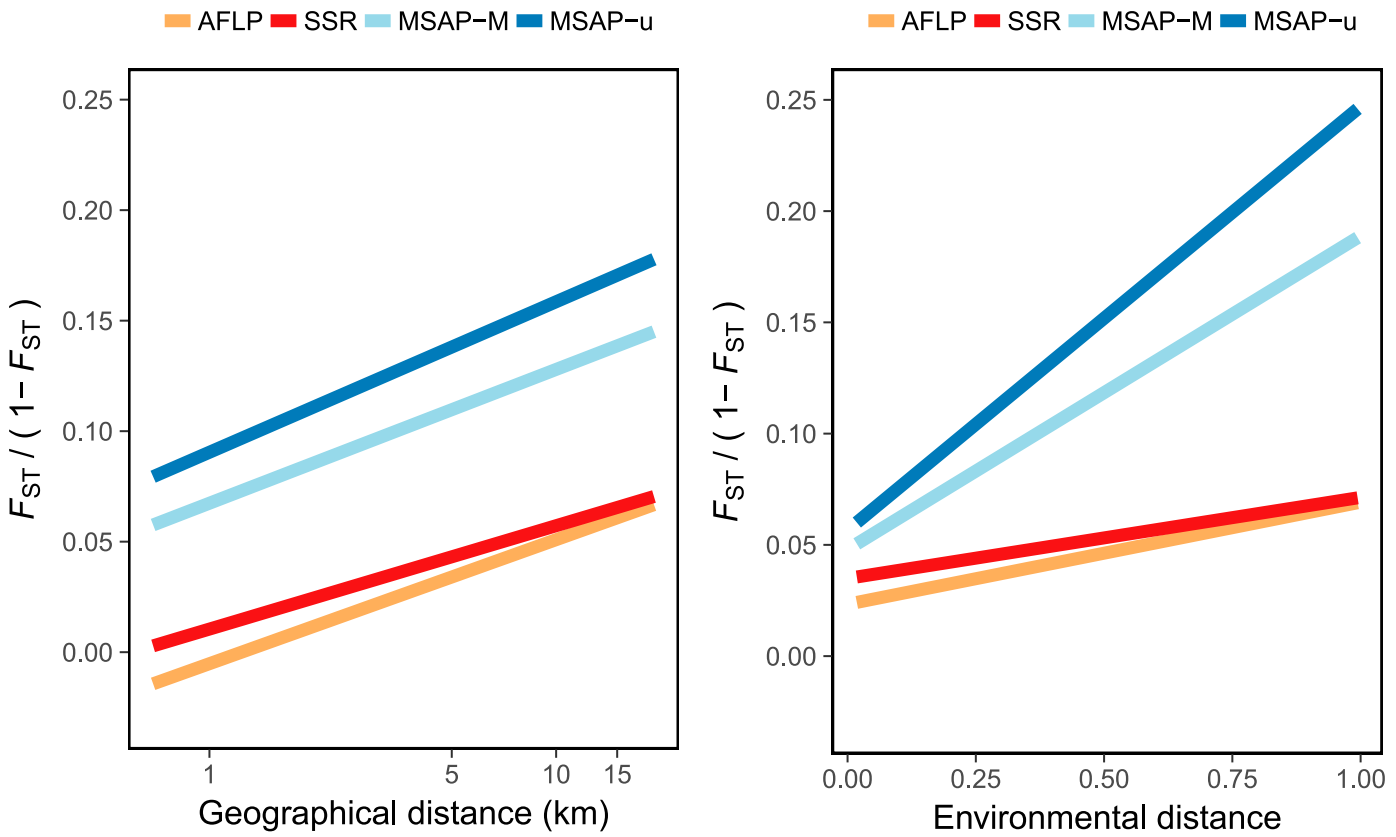


**FIGURE 1** Fitted linear regressions depicting the relationship between pairwise subpopulation differentiation [$F_{ST}/(1 - F_{ST})$] for genetic (AFLP and SSR; coded in shades of red) and epigenetic (MSAP *M*- and *u*-type; coded in shades of blue) markers, and the matrices of pairwise geographical (left; note logarithmic scale) and environmental (right) distance between subpopulations. See "Simple test" columns in Table 3 for statistical significance. See Appendices S3 and S4 for plots of regressions with data points.

**TABLE 4.** Results of multiple matrix regression analyses with randomization (MMRR) relating genetic and epigenetic differentiation matrices [$F_{ST}$ /(1 − $F_{ST}$)] between subpopulations of *Helleborus foetidus* with matrices of geographical and environmental distance. All matrices were scaled and centered before the analyses to obtain comparable linear coefficients.

| | | | Linear predictor matrices | | | |
|---|---|---|---|---|---|---|
| | Overall regression | | Geographical distance | | Environmental distance | |
| Differentiation matrix | *F* | *P* | Coefficient | *P* | Coefficient | *P* |
| Genetic | | | | | | |
| AFLP | 10.27 | 0.0003 | 0.443 | 0.0043 | 0.306 | 0.027 |
| SSR | 6.38 | 0.0012 | 0.352 | 0.0033 | 0.258 | 0.070 |
| Epigenetic | | | | | | |
| MSAP *M*-type | 1.83 | 0.074 | 0.122 | 0.37 | 0.282 | 0.12 |
| MSAP *u*-type | 4.80 | 0.0012 | 0.218 | 0.042 | 0.406 | 0.0093 |

*Notes: P* values were obtained by randomization with $10^5$ repetitions.

epigenetic differentiation between subpopulations, again irrespective of marker type. In fact, in the case of epigenetic differentiation the explanatory value of environmental distance was slightly superior to that of geographical distance (Fig. 2). These results were corroborated by MMRR analyses. In the case of genetic differentiation (AFLP and SSR), linear coefficients for the geographical distance matrix exceeded those for the environmental distance matrix, while the reverse held true for epigenetic differentiation (MSAP *M*- and *u*-type markers) (Table 4).

***Epigenetic IBE and phenotypic divergence***—In the MMRR analysis (Table 5), genetic distance was based on AFLP markers alone, as SSR patterns were nearly identical, and epigenetic distance was based on MSAP *u*-type markers alone because M-type markers showed no IBE. The regression was statistically significant, and there were statistically significant or marginally significant linear relationships between phenotypic distance and both environmental distance and epigenetic distance. In contrast, effects of geographical and genetic distances on phenotypic distance were not statistically significant (Table 5).

## DISCUSSION

***Subpopulation differentiation and isolation by distance***—The subpopulations studied were differentiated genetically and epigenetically. The $F_{ST}$ values computed from epigenetic markers doubled those for genetic ones, which suggests greater epigenetic than genetic differentiation as frequently found in other plants (Lira-Medeiros et al., 2010; Richards et al., 2012; Zhao et al., 2014; Huang et al., 2015). Under strict IBD, all neutral genomic markers are expected to display identical $F_{ST}$; hence, heterogeneous $F_{ST}$ values for different markers would provide evidence that systematic pressures have affected some but not others (Lewontin and Krakauer, 1973). By the same reasoning, increased $F_{ST}$ of epigenetic markers relative to genetic ones would provide evidence of continued pressures having enhanced epigenetic divergence between subpopulations above the level expected from the genetic $F_{ST}$ baseline, although similar patterns could possibly arise as a consequence of plastic epigenetic responses to variable environments.

This study has shown that the individual-level, small-scale genetic and epigenetic IBD reported previously for *H. foetidus* (Herrera et al., 2016) also holds at the between-population level. The positive and monotonically increasing relationships between genetic differentiation and geographical distance originate near the plot origin (Appendix S3) and are close to the case I differentiation–distance relationship in the classification of Hutchison and Templeton (1999) except for the absence of an increase in scatter with increasing distance, which might be attributed to the small geographical scale of this study. These patterns possibly reflect an equilibrium between gene flow and genetic drift, which would warrant inferences on the processes that have generated current spatial patterns of genetic and epigenetic diversity (Slatkin, 1993), as well as using population-level genetic IBD as a null model against which to compare the corresponding epigenetic patterns (Herrera et al., 2016). At equilibrium, and given that epigenetic marks will disperse in the same vehicles as genes (pollen and seeds) and should therefore be equally affected by migration–drift balance, epigenetic IBD across subpopulations should be identical to the corresponding genetic IBD unless they are disrupted by factors that act specifically on epigenetic variation (Slatkin, 1987; Herrera et al., 2016). Discrepancy between genetic and

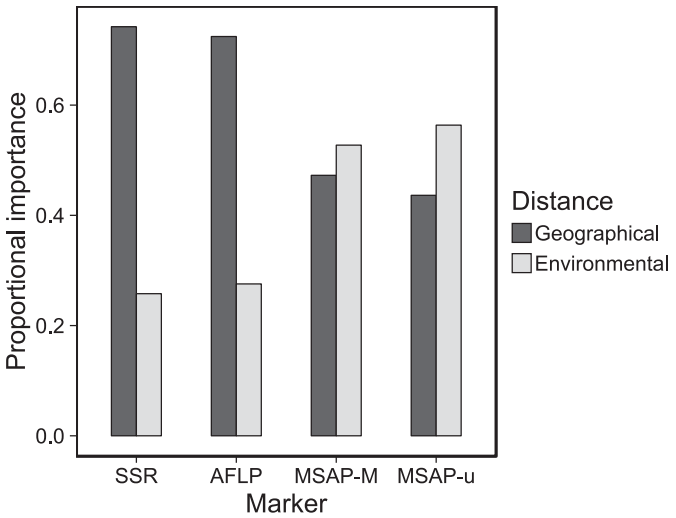


**FIGURE 2** Proportional importance of geographical and environmental distance as predictors of genetic (AFLP and SSR markers) and epigenetic (MSAP *M*- and *u*-type markers) differentiation [$F_{ST}$/(1 − $F_{ST}$)] between *Helleborus foetidus* subpopulations. Importance values were obtained by regressing genetic and epigenetic differentiation matrices against geographical and environmental distance matrices and then decomposing the $R^2$ of each regression into additive components estimating the relative importance of each predictor.

**TABLE 5.** Results of multiple matrix regression analysis with randomization (MMRR) relating the phenotypic distance matrix between subpopulations of *Helleborus foetidus* with the matrices of geographical, environmental, genetic (AFLP), and epigenetic (MSAP *u*-type markers) distances.

| | | Predictor matrices | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Overall regression | | Geographical distance | | Environmental distance | | Genetic distance | | Epigenetic distance | |
| *F* | *P* | Coefficient | *P* | Coefficient | *P* | Coefficient | *P* | Coefficient | *P* |
| 6.52 | 0.0039 | −0.083 | 0.48 | 0.188 | 0.059 | 0.090 | 0.57 | 0.301 | 0.024 |

*Notes:* All matrices were scaled and centered prior to the analyses. *P* values were obtained by randomization with $10^5$ repetitions to obtain comparable linear coefficients.

epigenetic IBD will therefore be informative on the operation of such disruptive factors.

Two factors can uniquely make epigenetic IBD depart from genetic IBD for the same subpopulations, namely, the tendency of epigenetic marks to be imperfectly transmitted across generations (Johannes and Colomé-Tatché, 2011; Schmitz et al., 2011; Herrera et al., 2013; Cortijo et al., 2014) and their capacity to experience modifications in response to the environment (Boyko and Kovalchuk, 2011; Zhang et al., 2013). Extensive resetting of parental epigenetic marks over successive generations should be equivalent to very high mutation rates, which would make the appearance of any epigenetic IBD pattern highly unlikely. Conversely, infrequent transgenerational resetting of epigenetic marks should produce epigenetic IBD closely resembling genetic IBD. At the limit, and in absence of plastic responses to the environment, perfect epigenetic inheritance should produce epigenetic IBD indistinguishable from genetic IBD. The approximately parallel differentiation–distance regressions for epigenetic and genetic markers found here for *H. foetidus* thus point to both limited plastic responses to the environment and limited transgenerational resetting of epigenetic marks in this species. The latter agrees with high sporophyte-to-gametophyte transmissibility of MSAP markers shown by Herrera et al. (2013, 2014). On the other hand, local epigenetic adaptation combined with plastic responsiveness of epigenetic marks to environmental factors could amplify epigenetic divergence relative to strict genetic IBD purely driven by migration–drift balance (Herrera and Bazaga, 2016; Herrera et al., 2016). As discussed below, differences between genetic and epigenetic spatial structure revealed by the current study can be interpreted in the context of the preceding framework.

***Isolation by environment: Patterns***—Consideration of environmental characteristics of *H. foetidus* subpopulations allowed us to assess whether epigenetic and genetic IBE were associated with geographical distance itself or were instead an indirect outcome of covariation between geographical and environmental distance across sites (Sexton et al., 2014, 2016; Durka et al., 2017). Partial Mantel tests and MMRR analyses similarly revealed an important contrast between genetic and epigenetic spatial patterns. While genetic differentiation between subpopulations was best explained by consideration of geographical distance alone, as found in many other plants (Diniz-Filho et al., 2013; Sexton et al., 2014; Durka et al., 2017), environmental distance played a major role in explaining epigenetic differentiation. This result is consistent with unrelatedness of genetic and epigenetic differentiation found here and in other plants (Li et al., 2008; Paun et al., 2010; Huang et al., 2015) and suggests that genetic and epigenetic spatial patterns in *H. foetidus* reflected contrasting causal processes. It must be stressed, however, that both genetic and epigenetic variation exemplified combined scenarios where IBD and IBE simultaneously applied. The disparity between the two classes of genomic variation arose from the difference in relative importance of geographical and environmental distances as predictors of subpopulation differentiation.

Results of this study agree with the prediction that spatial epigenetic structure is likely to conform frequently to IBE (Herrera et al., 2016). Correlations between environmental factors and epigenetic characteristics of plant populations have often been reported (Lira-Medeiros et al., 2010; Schulz et al., 2014; Huang et al., 2015; Foust et al., 2016; Keller et al., 2016), but these studies generally did not explicitly address the spatial component of the epigenome–environment relationship as done here. Despite their correlative nature, simultaneous spatial analyses of epigenetic, genetic, and environmental variation between populations are useful to evaluate the emerging, but still insufficiently documented notion that local environmental features can drive epigenetic differentiation of plant populations (Schulz et al., 2014; Foust et al., 2016; Wilschut et al., 2016).

***A hypothesis on epigenetic isolation by environment***—We suggest that essentially the same processes that explain genetic IBE, and particularly "isolation by adaptation" (Orsini et al., 2013; Wang and Bradburd, 2014), may contribute to generate epigenetic IBE. Since epigenetic variation can be transmitted across generations (Jablonka and Raz, 2009; Johannes et al., 2009), epigenetic IBE could arise as a consequence of native epigenotypes in each environment having higher average fitness than immigrant epigenotypes coming from other environments and hybrid offspring of immigrant-native crosses (Wang and Bradburd, 2014). Furthermore, exposure of immigrants to new environmental conditions could also trigger the release of novel epigenetic variants that could "explore" the new environmental space (Jablonka, 2013) and fitness landscape (Klironomos et al., 2013) more effectively, speeding up the process of local epigenetic adaptation under certain circumstances (Kronholm and Collins, 2016; see also Geoghegan and Spencer, 2012, 2013; Schlichting and Wund, 2014).

Support exists for some elements of this hypothesis. Particularly well supported are the prevalence of extensive but imperfect transgenerational inheritance of epigenetic marks that are often causally related to phenotypic traits (Jablonka and Raz, 2009; Verhoeven et al., 2010; Schmitz et al., 2011; Scoville et al., 2011; Cortijo et al., 2014) and the appearance of heritable epigenetic changes following exposure to environmental stress (Boyko and Kovalchuk, 2011; Kou et al., 2011; Tricker et al., 2013; Alonso et al., 2016). The latter would be equivalent to the environmental stress experienced by immigrants after arrival at a new habitat. Less frequently documented are putative local epigenetic adaptation (Dubin et al., 2015; Preite et al., 2015; Foust et al., 2016; Keller et al., 2016; Wilschut et al., 2016) and the release of new epigenetic variants following colonization of new environments (Richards et al., 2012; Herrera and Bazaga, 2016). Next to nothing is known about the possible adaptive

consequences of new, environmentally triggered epigenetic variants beyond inferences from theoretical models (Klironomos et al., 2013; Kronholm and Collins, 2016).

***Epigenetic IBE and phenotypic divergence***—Differentiation in functional traits among subpopulations of *H. foetidus* was associated with environmental and epigenetic divergence, but not with geographical and genetic distance. This correlative evidence suggests that phenotypic divergence was, at least in part, environmentally and epigenetically driven. This possibility is supported by previous studies demonstrating associations among traits and MSAP markers, and spatially variable selection in *H. foetidus* (Herrera et al., 2014; Medrano et al., 2014). Associations between markers and traits across individuals are not a proof of causality, but experimental studies on model plants have often found causal links between epigenetic variation and individual differences in functional traits (Cubas et al., 1999; Akimoto et al., 2007; Johannes et al., 2009; Scoville et al., 2011). These causal links lend plausibility to the interpretation that some epigenetic marker–trait associations in *H. foetidus* might reflect a causal relationship and that the epigenetic loci involved could be susceptible to selection. In this hypothesized scenario, spatially variable selection reported for *H. foetidus* (Rey et al., 2006a; Herrera et al., 2014) in combination with imperfect epigenetic inheritance would eventually produce epigenetically based adaptive phenotypic divergence of the sort predicted by theoretical models (Uller et al., 2015; Kronholm and Collins, 2016). Experimental work is needed to verify these views.

## CONCLUDING REMARKS

Understanding the ecological causes and consequences of epigenetic variation in natural populations was recently singled out as a fundamental ecological question (Sutherland et al., 2013). A satisfactory answer will require a deeper understanding of the patterns and adaptive significance of epigenetic variation between individuals and populations, which will in turn depend on improved knowledge about the extent to which such variation is environmentally driven, autonomous from genetic variation, inherited across successive generations, and causally related to functional traits. Orsini et al. (2013) advocated for investigations that account for the role of space as well as environment on both neutral and nonneutral genetic variations. Wang and Bradburd (2014) likewise noted that comparisons between IBE and IBD could contribute to our understanding of the types of gene flow that enable population divergence. Results of the present investigation, along with the fact that epigenetic and genetic variation can often act as independent layers of heritable variation in plants (Richards, 2006; Johannes et al., 2009; Herrera and Bazaga, 2010; Johannes and Colomé-Tatché, 2011), suggest that contrasting the relative effects of geographical and environmental variation on genetic, epigenetic, and phenotypic variation can help to unravel the ecological and evolutionary significance of epigenetic variation.

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