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EPIGENETIC STUDIES IN ECOLOGY AND EVOLUTION

Comparative spatial genetics and epigenetics of plant populations: heuristic value and a proof of concept

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Abstract

Despite the recent upsurge of interest on natural epigenetic variation of nonmodel organisms, factors conditioning the spatial structure of epigenetic diversity in wild plant populations remain virtually unexplored. We propose that information on processes shaping natural epigenetic variation can be gained using the spatial structure of genetic diversity as null model. Departures of epigenetic isolation-by-distance (IBD) patterns from genetic IBD patterns for the same sample, particularly differences in slope of similarity-distance regressions, will reflect the action of factors that operate specifically on epigenetic variation, including imperfect transgenerational inheritance and responsiveness to environmental factors of epigenetic marks. As a proof of concept, we provide a comparative analysis of spatial genetic and epigenetic structure of 200 mapped individuals of the perennial herb *Helleborus foetidus*. Plants were fingerprinted using nuclear microsatellites, amplified fragment length polymorphisms (AFLP) and methylation-sensitive AFLP markers. Expectations from individual-level IBD patterns were tested by means of kinship-distance regressions. Both genetic and epigenetic similarity between *H. foetidus* individuals conformed to theoretical expectations under individual-level IBD models. Irrespective of marker type, there were significant negative linear relationships between the kinship coefficient for plant pairs and their spatial separation. Regression slopes were significantly steeper for epigenetic markers. Epigenetic similarity between individuals was much greater than genetic similarity at shortest distances, such epigenetic 'kinship excess' tending to decrease as plant separation increased. Results suggest that moderate-to-high heritability and responsiveness to local environments are major drivers of epigenetic spatial structure in *H. foetidus*, and illustrate the heuristic value of comparing genetic and epigenetic spatial structure for formulating and testing hypotheses on forces shaping epigenetic diversity in wild plant populations.

Keywords: DNA methylation, epigenetic diversity, genetic diversity, *Helleborus foetidus*, isolation by distance, spatial structure

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Introduction

Theoretical and empirical investigations on the spatial distribution of genetic diversity in natural populations have remained for decades in the focus of population genetics and evolutionary biology (Wright 1943, 1978; Malécot 1948; Endler 1977; Epperson 2003). Factors driving the spatial structure of genetic diversity in natural plant populations, as well as patterns associated with different life forms, breeding systems, dispersal traits and ecological scenarios, are thus nowadays reasonably well understood (Charlesworth *et al.* 2003; Vekemans & Hardy 2004; Hardy *et al.* 2006; Sexton *et al.* 2014). This provides a privileged starting point for undertaking comparative analyses of the spatial structure of epigenetic diversity in plant populations, an aspect that remains largely unexplored despite the

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recent upsurge of interest on the magnitude, patterns and implications of natural epigenetic variation in non-model plants (Herrera & Bazaga 2010; Medrano *et al.* 2014; Schulz *et al.* 2014; Preite *et al.* 2015). Comparisons of the genetic and epigenetic spatial structure of populations of the same species are of considerable interest, as 'spatial patterns capture the cumulative effects of forces acting over many generations, [and] it may be possible to use them to study the presence and operation of these forces' (Epperson 2003).

In this study, we will first briefly review what is currently known on the comparative spatial structure of genetic and epigenetic diversity in natural plant populations. Second, we will introduce a conceptual framework positing that the spatial structure of genetic diversity may be used as a null model to generate hypotheses and obtain retrospective information on the processes shaping epigenetic variation in natural populations. We argue that comparisons of the spatial patterns of genetic and epigenetic variation for the same sample of individuals may help to understand some elusive aspects of epigenetic variation in natural plant populations that remain poorly known, such as long-term transgenerational stability of epigenetic characteristics or the role played by environment factors in the epigenetic differentiation of local populations (Richards 2008, 2011). And third, a comparative analysis of spatial genetic and epigenetic structure will be presented for the perennial herb *Helleborus foetidus* as a proof of concept. It will be beyond the scope of this study to dwell on the causal factors that determine the details of spatial genetic patterns in plants. Such patterns will be treated here only as an empirical 'baseline reference', or 'null model', with which to compare spatial epigenetic patterns.

Spatial structure of genetic and epigenetic diversity in plants

The few studies that have so far compared genetic and epigenetic structure in wild plants have consistently shown that, like genetic diversity, epigenetic diversity may also be spatially structured at various scales (Table S1, Supporting information). Furthermore, spatial epigenetic differences between conspecific populations often exceed their genetic differences (Table S1, Supporting information) and sometimes are predictably related to variation in environmental factors (Lira-Medeiros *et al.* 2010; Richards *et al.* 2012; Schulz *et al.* 2014). In cases where intraspecific epigenetic differences persist across generations (Jablonka & Raz 2009; Herrera *et al.* 2013), such spatial structure might reflect adaptive epigenetic divergence independent of genetic differences (Herrera & Bazaga 2010; Lira-Medeiros *et al.* 2010; Herrera *et al.* 2014; Schulz *et al.* 2014), which further highlights the interest of elucidating patterns and mechanisms of natural epigenetic variation.

When results of the handful of investigations conducted to date on spatial structuring of natural epigenetic diversity are placed in a common spatial context, two hitherto unrecognized patterns emerge as follows: epigenetic differences between conspecific populations tend to increase with the spatial scale of the study (= geographical distance between most distant locations sampled), and the slopes of difference–distance relationships are approximately similar for epigenetic and genetic measurements (Fig. 1). The data plotted in Fig. 1 are few and not strictly comparable, as they refer to different species. This caveat notwithstanding, however, the positive relationship between epigenetic difference and spatial distance is reminiscent of isolation-by-distance (IBD hereafter) patterns often exhibited by genetic variation in natural plant populations (Heywood 1991; Epperson 2003; Sexton *et al.* 2014). This prompts the hypothesis that spatial structure of epigenetic diversity in plants may be driven, at least in part, by the same main factor driving spatial structuring of genetic variation, namely limited gene dispersal via

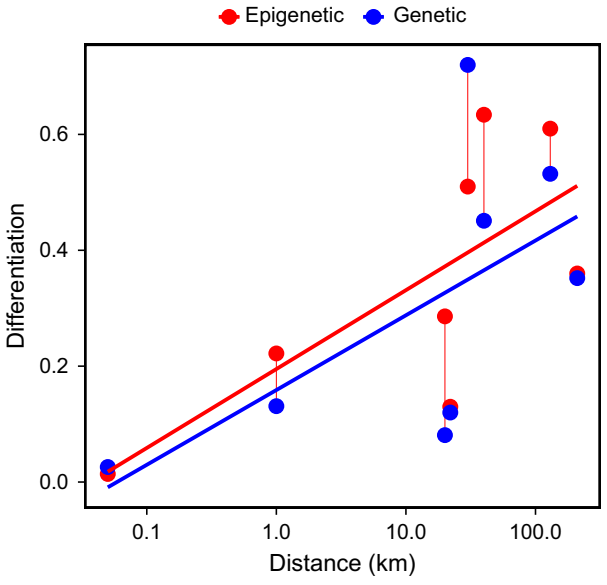

Fig. 1 Relationship between spatial scale (distance between most distant sampling locations) and multilocus genetic and epigenetic differentiation (Φ_{ST}) between populations of eight plant species, as obtained from published information. Vertical segments join the pair of data points for the same species, and the two lines are least-squares regressions fitted separately to genetic and epigenetic data. Data for the graph were taken from studies providing simultaneous estimates of genetic and epigenetic differentiation for populations of the same species (Table S1, Supporting information).

pollen and seeds (Wright 1943; Epperson 2003; Vekemans & Hardy 2004).

We propose in the next section that empirical IBD patterns of genetic variation can be used as null models with which to compare the corresponding spatial patterns of epigenetic variation and that various forms of departure of epigenetic IBD relative to the genetic null model of reference can inform on the processes potentially shaping the spatial structure of epigenetic diversity. Comparative tests of IBD models for genetic and epigenetic data for a given sample of mapped individuals of the same species can therefore be of heuristic value to suggest hypotheses and obtain insights on the forces shaping natural epigenetic diversity. Two major types of spatial genetic distributions and IBD patterns have been traditionally recognized as follows: 'individual-level' models, in which the distribution of individual genotypes within a single, large population is considered, and 'population-level' models, which consider spatial variation of gene frequencies among discrete populations (Epperson 2003). Despite differences in type of data, analytical methods and details of the demographic processes involved, individual- and population-level models have a fundamental conceptual similarity, namely the prediction that genetic similarity between individuals will decline (individual-level), and genetic differentiation between populations will increase (population-level), with increasing distance between entities (individuals or populations). For simplicity, the conceptual model introduced in the next section will be framed in individual-level terms alone, and only individual IBD models will be considered throughout this study. Nevertheless, it should not be difficult in most instances to 'translate' our individual-level treatment, *mutatis mutandi*, into a population-level framework. Individual-level models treat all individuals as belonging to a single population, and their application to the *H. foetidus* case study is justified by the essentially continuous distribution of the species over the area sampled.

Comparative genetic and epigenetic isolation-by-distance patterns

Individual-level IBD models predict a monotonous decline in pairwise genetic similarity between individuals (estimated using, e.g. kinship or relatedness measurements) with increasing spatial separation, and such relationship has been corroborated empirically in most species where it has been looked for (Heywood 1991; Epperson 2003; Vekemans & Hardy 2004; Hardy *et al.* 2006; Meirmans 2012; Ley & Hardy 2013). In two-dimensional populations under limited gene dispersal, the genetic similarity between individuals is expected to decline linearly with increasing logarithm of distance (Rousset 1997, 2000), as in the blue lines of panels 1–6 in Fig. 2. The slope of the similarity-log(distance) regression reflects the rate of decrease of pairwise similarity between individuals with the logarithm of the distance, will be inversely related to the magnitude of gene flow by pollen and seeds and can be used to quantify the extent of spatial genetic structure (Hardy & Vekemans 2002; Vekemans & Hardy 2004). Whichever the extent of gene dispersal in a given population, however, genomic epigenetic marks (e.g. methylation status of individual cytosines) or epialleles (Kalisz & Purugganan 2004; Weigel & Colot 2012) will disperse in the same vehicles as genes (pollen and seeds) and will experience identical dispersal patterns. At equilibrium with respect to contemporary population dynamics, therefore, the individual-level epigenetic IBD pattern of a given population should be identical to the corresponding individual-level genetic IBD pattern, *unless it is disrupted by factors that act specifically on epigenetic variation alone*. Discrepancies between genetic and epigenetic IBD patterns in a population will thus provide information on the operation of such epigenetic-specific disrupting factors.

Two defining features that set epigenetic variation apart from genetic variation in current usage (Richards 2006; Jablonka 2013) are as follows: (i) the propensity of epigenetic marks to be imperfectly transmitted across generations (Schmitz *et al.* 2011; Herrera *et al.* 2014; Li *et al.* 2014; Avramidou *et al.* 2015; Van der Graaf *et al.* 2015) and (ii) their capacity to exhibit short-term modifications in response to environmental factors (Boyko *et al.* 2007; Boyko & Kovalchuk 2011; Hauser *et al.* 2011; Rico *et al.* 2014; Alonso *et al.* 2016). Obviously, none of these apply to genetic variation (i.e. DNA sequence variants). We therefore contend that departures of epigenetic IBD patterns relative to genetic IBD ones in the form of differential similarity-distance relationship could be interpreted in terms of these two major epigenetic-specific factors. The disruptive effect of imperfect inheritance on epigenetic IBD patterns should be qualitatively equivalent to that of high mutation, which is known to reduce the extent of spatial genetic structure (Wright 1978; Epperson 2003, 2005; Leblois *et al.* 2003; Ley & Hardy 2013). Everything else being equal, low or negligible transgenerational constancy of epigenetic marks (equivalent to very high mutation rate) should tend to flatten epigenetic similarity-distance regressions relative to genetic ones, whereas high or perfect transgenerational transmission will make the slopes of epigenetic regressions to converge towards those of genetic regressions. Responsiveness of epigenetic marks to environmental factors can take the form of short-term plastic responses, long-term selection on heritable markers

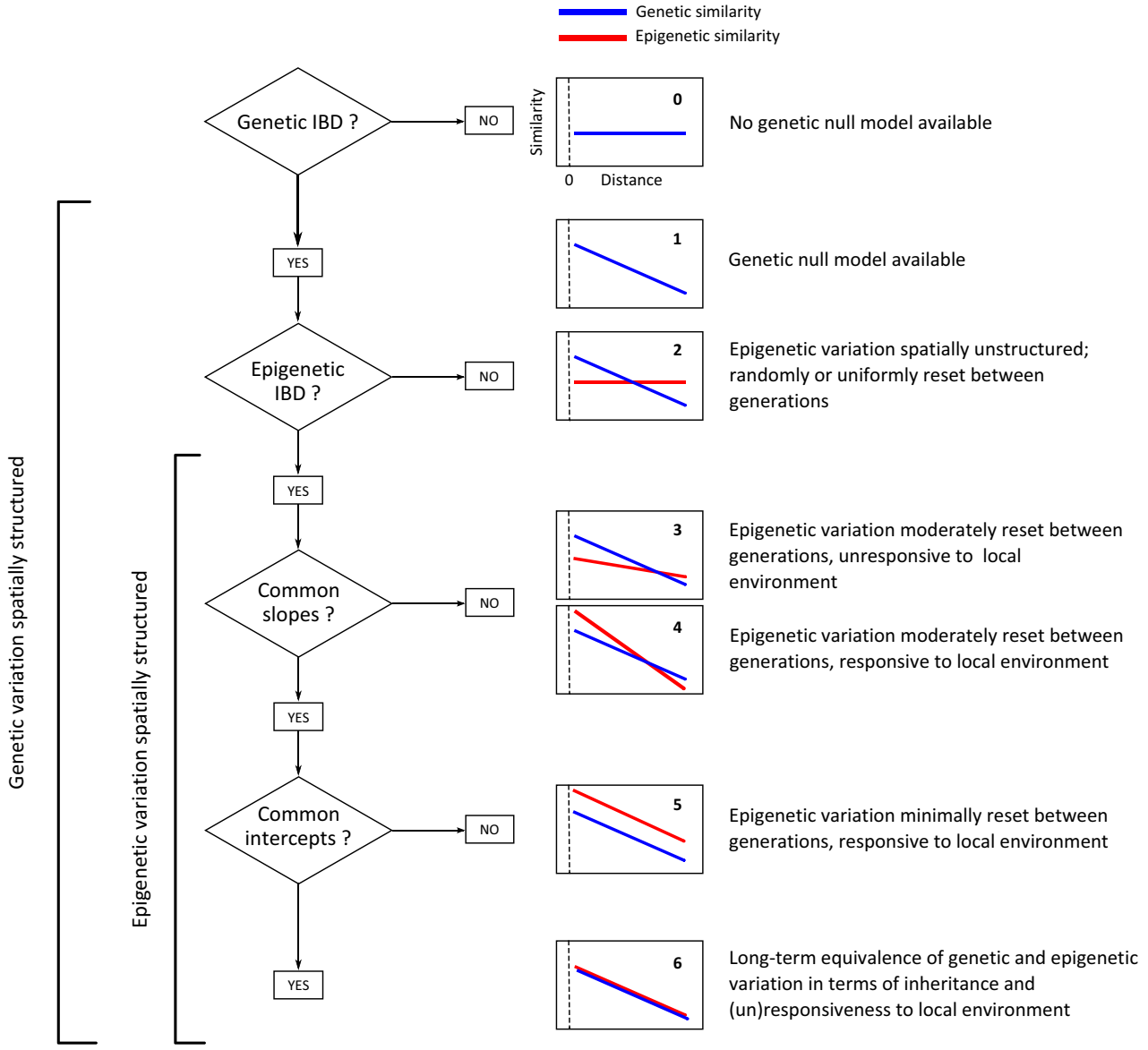

Fig. 2 Flow diagram illustrating some possible genetic and epigenetic isolation-by-distance (IBD) scenarios in natural plant populations, along with hypothesized causes. Alternative interpretations of some of the patterns depicted could be possible. Lines depict the relationships between pairwise genetic (blue) and epigenetic (red) similarity between individuals and their spatial separation. Whenever an inverse relationship between genetic similarity and distance exists in a given population, depicted by the same blue line in panels 1–6, the regression slope should mainly reflect the extent of gene dispersal. As epigenetic variation will be subject to identical dispersal processes as genetic variation, the genetic IBD pattern provides a null model of reference with which to compare the relationship between epigenetic similarity and distance. When significant epigenetic IBD also exists, differences in slopes (panels 3–4) and intercepts (only in the case of common slopes; panels 5–6) between the lines depicting genetic and epigenetic IBD patterns for the same sample of individuals are hypothesized to reflect the action of factors that operate exclusively on epigenetic variation, such as imperfect inheritance and responses to environmental factors.

or some combination of these (Shea *et al.* 2011). Irrespective of the mechanism(s) involved, coordinated spatial variation of environment and epigenetic states (Furrow *et al.* 2011) will ultimately translate into a disproportionate increase in epigenetic similarity (i.e. an apparent epigenetic 'similarity excess' relative to purely kinship-driven genetic similarity) of plants growing within short distances and exposed to presumably similar environments. Such effects should lead to a reduction of epigenetic patch size relative to genetic patch size, defined operationally as the *x*-intercept of the respective similarity-distance relationships (Epperson

2003). Under limited dispersal, directional selection is predicted to reduce the patch sizes and spatial correlations of genetic variation (Epperson 1990), and similar effects on epigenetic spatial structure should be expected from directional selection on heritable epigenetic variation.

A stepwise comparison of genetic and epigenetic similarity-distance relationships for the same sample of individuals is presented in Fig. 2 as a flow diagram to illustrate possible scenarios differing in inheritance and environmental responsiveness of epigenetic variation. The graphs show different hypothetical situations we should expect to see when comparing epigenetic and genetic IBD patterns. The patterns depicted were selected for illustrative purposes only and represent a subset of the possibilities that can be envisaged. In addition, it should be noted that alternative interpretations of the patterns shown could be possible in some cases. Situations where genetic diversity is spatially structured according to IBD (significant similarity-distance regression) but epigenetic diversity is not (non-significant similarity-distance regression; Fig. 2, panel 2) may be explained by extensive and/or random reset of epigenetic marks between generations, leading to epigenetic marks of parents and offspring being largely uncorrelated (i.e. heritability effectively equal to zero). In contrast, when both genetic and epigenetic regressions are significant (Fig. 2, panels 3–6), it will be indicative of heritability of epigenetic marks being sufficiently high to produce the significant parent–offspring correlations required for IBD patterns to arise. In other words, simultaneous genetic and epigenetic IBD patterns within populations will support the long-term transgenerational inheritance of epigenetic marks. The four main situations that may be envisaged can be conveniently dissected by drawing an analogy to the usual procedure in conventional analysis of covariance, that is testing first for homogeneity of slopes of epigenetic and genetic regressions (Fig. 2, panels 3 and 4) and then, only if such homogeneity cannot be rejected, testing for homogeneity of intercepts (Fig. 2, panels 5 and 6). For example, moderate-to-low heritability of epigenetic marks would lead to situations similar to that of panel 4 if epigenetic variation is partly shaped by responses to local environmental factors and similar to panel 3 if epigenetic marks are largely unresponsive to environmental factors. High mean heritability of epigenetic marks coupled with high responsiveness to local environment factors may in the long run produce a pattern similar to panel 5, while close similarity of genetic and epigenetic variation with regard to inheritance and (un) responsiveness to environmental conditions will lead to virtually identical genetic and epigenetic IBD patterns as depicted in panel 6.

A case study: comparative spatial genetics and epigenetics of *Helleborus foetidus*

Helleborus foetidus L. (Ranunculaceae) is a perennial herb widely distributed in western and south-western Europe, occurring in diverse habitats ranging from open scrub to conifer and broad-leaved forests from sea level to 2100 m elevation (Mathew 1989). Flowers are hermaphroditic, self-compatible and nearly exclusively pollinated by bumblebees. Fruit maturation and seed shedding take place in June–early July. After falling to the ground, seeds either remain under the parent plant or are dispersed short distances by ants. Seedling recruitment beyond the close vicinity of parent plants is usually negligible (Manzaneda & Rey 2008; Herrera *et al.* 2014).

Twenty widely spaced, inflorescence-bearing plants were selected at each of ten locations in the Sierra de Cazorla (Jaén Province, south-eastern Spain). Clonal propagation of *H. foetidus* plants is exceptional and, when it occurs, it is spatially very limited (< 1 m) and easily discerned (Werner & Ebel 1994). We avoided sampling nearest neighbours whenever doubts arose on possible clonality, and it can be safely assumed that all plants sampled came from distinct genets. Young leaves were collected from each plant and dried at ambient temperature in containers with silica gel. Plants were georeferenced with a GPS receiver to the nearest 4–6 m (depending on satellite coverage). This accuracy was considered sufficient for the purposes of this study, as only 2.4% of pairwise distances between plants from the same site ($N = 1900$) were ≤6 m. Plants were the same studied by Medrano *et al.* (2014), where detailed information on location and ecological characteristics of sampling sites was provided.

Laboratory methods

All plants were characterized genetically and epigenetically. Total genomic DNA was extracted from dry leaf samples using Qiagen DNeasy Plant Mini Kit and the manufacturer protocol. Genetic and epigenetic analyses were conducted on the same DNA extracts. Genetic fingerprints were obtained for each plant using amplified fragment length polymorphism markers (AFLP; Weising *et al.* 2005; Meudt & Clarke 2007) and nuclear microsatellites (SSR hereafter). The AFLP analyses were performed using standard protocols involving the use of fluorescent dye-labelled selective primers (Weising *et al.* 2005) and a total of eight *Pst*I + 2/*Mse*I + 3 primer pairs. Individual AFLP fingerprints used here were taken from Medrano *et al.* (2014), where additional details on methods can be found. Plants were also genotyped using 11 polymorphic SSR loci [*Hefo1*, *Hefo2*,

Hefo3, *Hefo4*, *Hefo5*, *Hefo7*, *Hefo8*, *Hefo9*, *Hefo10*, *Hefo11*, *Hefo13*; details on amplification conditions and PCR cycle profiles may be found in Consortium MERPD *et al.* (2013)]. Amplified products were analysed on an ABI PRISM 3130xl DNA sequencer. Fingerprint profiles were scored by visualizing electrophoregrams with GENEMAPPER 3.7 software.

The methylation-sensitive amplified polymorphism technique (MSAP; Schulz *et al.* 2013; Fulneček & Kovařik 2014) was used to characterize plants epigenetically. MSAP is a modification of the standard AFLP technique that uses the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I in parallel runs in combination with another restriction enzyme. Differences in the products obtained with *Hpa*II and *Msp*I reflect different methylation states of cytosines in the CCGG sites recognized by *Hpa*II or *Msp*I (see Schulz *et al.* 2013; Fulneček & Kovařik 2014; for references and further details). MSAP assays were conducted using four *Hpa*II-*Msp*I + 2/*Mse*I + 3 primer combinations. Raw MSAP fragment data used here were the same analysed by Medrano *et al.* (2014).

Data analysis

Parameters describing the genetic spatial structure for a given data set may depend on the type of genetic marker selected (Hardesty *et al.* 2005; Hardy *et al.* 2006; Jump & Peñuelas 2007; Ley & Hardy 2013). Two different genetic markers were used here (SSR and AFLP) to obtain a robust assessment of the spatial structure of genetic diversity in *H. foetidus*. The use of dominant markers to test IBD models implies the adoption of allele frequency-based approaches (*sensu* Bonin *et al.* 2007), which in turn requires information on the inbreeding coefficient of sampled individuals (Zhivotovsky 1999; Hardy 2003; Ley & Hardy 2013). We estimated the inbreeding coefficient for *H. foetidus* plants in our sample from the codominant SSR data, and the figure obtained (F_{is} = 0.141) was then used in all analyses that required computations of allele frequencies from dominant marker data (AFLP and MSAP).

Unless otherwise stated, all statistical analyses were carried out using the R environment (R Development Core Team 2014). For simplicity, the 'Mixed Scoring 1' transformation scheme of Schulz *et al.* (2013) was applied to the two presence–absence matrices for MSAP fragments obtained with the four *Hpa*II-*Mse*I and *Msp*I-*Mse*I primer combination pairs. Under this scheme, MSAP fragments are transformed into two distinct sets of markers, corresponding to unmethylated and methylated types [*u* and *M* markers in Schulz *et al.*'s (2013) terminology, respectively]. All plants sampled were characterized epigenetically by means of the presence–absence scores for *u*-type (N = 105) and *M*-type (N = 142) MSAP markers, using the function Extract_MSAP_epigenotypes from Schulz *et al.* (2013) with parameters Epicode = 'Mix1', delete.monomorphic.loci = TRUE, and MinPoly = 2.

Two related methods were adopted to identify and compare individual-level IBD patterns of genetic and epigenetic variation, using version 1.4 of SPAGEDI for computations (Hardy & Vekemans 2002). The first method involved testing if, as predicted from theoretical models, there existed an inverse linear relationship between pairwise genetic/epigenetic similarity between individuals and the logarithm of their spatial separation (Rousset 2000; Vekemans & Hardy 2004; Guillot *et al.* 2009). The kinship coefficient between pairs of individuals was computed using Loiselle *et al.* (1995) coefficient for codominant SSR data, and the estimator proposed by Hardy (2003) for dominant AFLP and MSAP data. Following Leblois *et al.* (2003), information on allele size was not incorporated into kinship computations from SSR data. All individuals in the sample were used for defining reference allele frequencies. This regression-based method considers simultaneously all the data, that is without classifying pairwise distances into arbitrary distance intervals, and is fairly robust to various demographic, mutational and sampling factors (Rousset 1997, 2000; Guillot *et al.* 2009; Luximon *et al.* 2014). Estimates of regression slopes (β_i) were used to compare kinship-distance relationships for the different marker types and identify scenarios of the sort proposed in Fig. 2. Statistical significance of individual regression slopes (H_0: $\beta_i = 0$ vs H_A: $\beta_i < 0$) was assessed using permutation tests with 5000 repetitions. Approximate standard errors of slope estimates were computed by jackknifing over markers (Hardy & Vekemans 2002), and approximate 95% confidence intervals obtained as $\beta_i \pm 2SE$. Homogeneity of regression slopes for different marker types (H_0: $\beta_{SSR} = \beta_{AFLP} = \beta_{MSAP\text{-}M} = \beta_{MSAP\text{-}u}$) was tested using a simplified ANCOVA-like permutation approach. A linear model was fitted to the kinship-distance data for all marker types combined, with kinship as dependent variable and the marker type × log10(distance) interaction as the only independent effect. The *F*-value for the interaction thus obtained was then compared with the distribution of corresponding *F*-values obtained by fitting the same model to 10^5 simulated data sets with marker types randomly permuted.

To obtain further details on the differential patch size of genetic and epigenetic diversity, distogram analyses (e.g. Cavers *et al.* 2005; Hardesty *et al.* 2005) were also undertaken. This procedure involved assessing how the mean genetic/epigenetic similarity between individuals varied with increasing distance along a series of

predefined discrete intervals (e.g. Vekemans & Hardy 2004; Hardy *et al.* 2006; Ley & Hardy 2013). The range of individual pairwise distances occurring in our sample (0.003–19 km) was divided into 15 distance intervals each of which contained the same number of pairs. In separate analyses for different marker types, data subsets were constructed for each predefined distance interval that included all pairs of individuals separated by a distance falling within the class. Mean kinship coefficient for each distance interval was plotted against the mean distance for each class, and the distograms thus obtained were used to compare spatial structuring of genetic and epigenetic diversity (Guillot *et al.* 2009; Ley & Hardy 2013). Statistical significance of mean kinship for every distance interval was determined by comparing observed values with the corresponding frequency distributions obtained by random permutations of the data. Approximate standard errors of observed mean kinship coefficients were obtained by jackknifing over markers.

Results

Both genetic and epigenetic similarity between the *H. foetidus* individuals sampled conformed to theoretical expectations under individual-level IBD models. Irrespective of marker type, there were negative, statistically significant linear relationships between the kinship coefficient for plant pairs and their spatial separation (Table 1). Regression R^2 for the two types of MSAP markers were considerably higher than the corresponding figures for AFLP and SSR markers, suggesting a closer fit of epigenetic data to theoretical expectations under individual-level IBD models.

The null hypothesis of a common regression slope for the four marker types considered was conclusively rejected ($P < 10^{-5}$) by the permutation test [observed *F*-value for the marker × log10(distance) interaction = 2490; 100% percentile of 10^5 simulated *F*-values = 2271]. Overall heterogeneity of slopes was due to the marked contrast between genetic and epigenetic markers (Fig. 3). The 95% confidence intervals for AFLP and SSR slopes, on one side, and MSAP–M and MSAP–u slopes, on the other, did not overlap and were separated by a wide gap (Table 1). Slopes for the two types of MSAP markers were considerably steeper than those for the two types of genetic markers (Table 1, Fig. 3). As a consequence, kinship estimates obtained with epigenetic markers were much greater than those for genetic markers only for the closest plant pairs (<~100 m), and such epigenetic 'kinship excess' tended to decrease as plant separation increased (Fig. 3; see also Fig. S1, Supporting information). Within each marker class (genetic and epigenetic), the two 95% confidence intervals corresponding to the two marker types did overlap (Table 1), which suggests class-specific IBD patterns irrespective of the particular marker considered (AFLP and SSR on one side, MSAP-M and MSAP-u on the other).

Results of distogram analyses corroborated those of kinship-distance regressions. The global shape of distograms was similar for all marker types, with kinship relationships between plant pairs being significantly

Table 1 Regression-based tests of genetic and epigenetic isolation-by-distance patterns in the sample of $N = 200$ plants of *Helleborus foetidus* studied. Regression lines are plotted on a common axis frame in Fig. 3 (see also Fig. S1, Supporting information)

	Pairwise kinship vs log10(distance) regression			
	Slope (β_i)			
Marker type	Estimate	95% Confidence Interval	*P*-value (H_0: $\beta = 0$)	R^2
Genetic				
SSR	−0.0350	−0.0444 to −0.0256	<0.0002	0.036
AFLP	−0.0257	−0.0295 to −0.0219	<0.0002	0.089
Epigenetic				
MSAP *M*-type	−0.0690	−0.0846 to −0.0534	<0.0002	0.198
MSAP *u*-type	−0.0745	−0.0979 to −0.0511	<0.0002	0.150

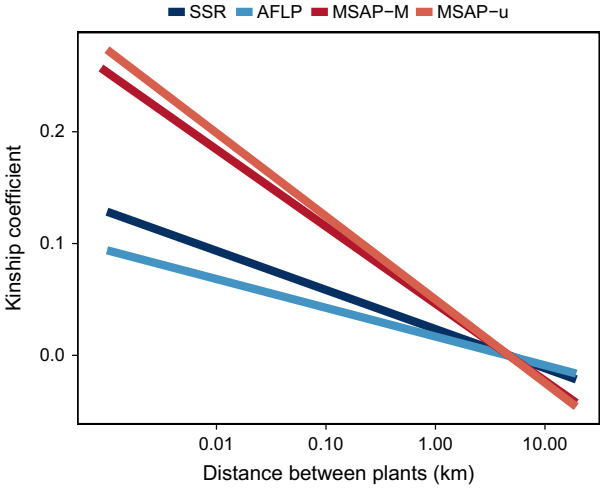

Fig. 3 Fitted linear regressions for the genetic (SSR and AFLP) and epigenetic markers (MSAP *M*-type and *u*-type), depicting the relationships between individual pairwise kinship coefficient and spatial separation (note logarithmic scale) for the $N = 200$ plants of *Helleborus foetidus* sampled. See Table 1 for slope estimates and significance tests, and Fig. S1 (Supporting information) for regression plots of actual data points.

higher than expected only in the first few distance classes (Fig. 4). Genetic patch size, as estimated from distograms for AFLP and SSR markers, was ~5 km. Estimated size of epigenetic patches was considerably narrower (~1.5 km) regardless of MSAP marker type (Fig. 4), thus revealing a stronger spatial structuring of epigenetic variation.

Discussion

Individual-level analyses of *H. foetidus* data revealed significant genetic IBD patterns regardless of the marker used. Kinship-distance regression slopes for AFLP and SSR data were statistically indistinguishable, and the proportion of sample variance in pairwise kinship values explained by spatial distance (regression R^2) was approximately similar for the two marker types (Table 1). This contrasts with some previous reports of differences between these markers in their ability to detect spatial structure (Gaudeul *et al.* 2004; Hardesty *et al.* 2005; Jump & Peñuelas 2007). Given the high scoring error rates sometimes associated with AFLP markers (Bonin *et al.* 2004) and the depressing effect of low marker repeatability on estimates of IBD parameters (Ley & Hardy 2013), failures of AFLP data to detect spatial genetic structure (e.g. Hardesty *et al.* 2005) might sometimes stem from insufficient control of marker quality. And conversely, the close concordance found here for AFLP and SSR markers (see also Hardy 2003) may be attributed to their similarly high repeatabilities in our sample (Medrano *et al.* 2014; C. M. Herrera and M. Medrano, unpublished). In the context of the present study, congruence of IBD regression slopes for AFLP and SSR markers hold up the assumption that they reflect patterns of gene dispersal in *H. foetidus*, hence warranting their use as a null model with which to compare epigenetic IBD regression slopes.

Like genetic diversity, epigenetic diversity of *H. foetidus* was also spatially structured in the area studied. Individual-level regression and distogram analyses revealed significant epigenetic IBD, with pairwise kinship between plants declining significantly with increasing distance. IBD patterns were similar for the two MSAP marker types considered. Goodness-of-fit of kinship-distance regressions, as assessed with the regression R^2, was considerably higher for epigenetic than for genetic markers (Table 1), thus indicating that distance between plants was a better predictor of epigenetic than genetic similarity. While epigenetic differences between conspecific plant populations have been demonstrated in recent years nearly whenever it was sought (Ma *et al.* 2013; Yu *et al.* 2013; Huang *et al.* 2015; and references in Table S1, Supporting information), results presented here for *H. foetidus* seem the first evidence to date of epigenetic IBD for a wild plant.

The slopes of kinship-distance regressions were considerably steeper for epigenetic than genetic markers. The conclusion from distogram analyses was similar. Only at the shortest distances, which mostly but not exclusively correspond to plant pairs from the same

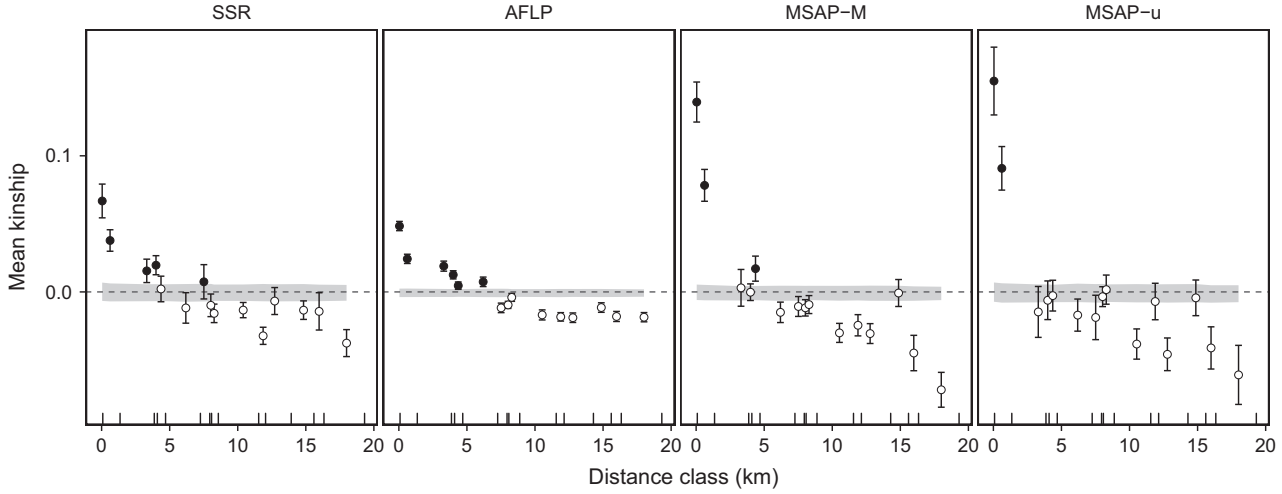

Fig. 4 Mean kinship coefficients between individuals (dots) for different distance intervals in the sample of *Helleborus foetidus* plants studied, assessed using genetic (AFLP, SSR) and epigenetic markers (MSAP *M*-type and *u*-type). Vertical segments around means represent ± 1 SE obtained by a jackniffing procedure over loci. Filled and empty dots represent, respectively, mean kinships significantly ($P < 0.01$) and nonsignificantly greater than the mean obtained when data in a given distance class were randomly permuted. The shaded area around the dashed $y = 0$ reference line represents the two-sided 95% confidence interval for randomly generated mean kinship coefficients. Each of the $N = 15$ distance intervals chosen contained the same number of pairs of individuals. Rug plots on the horizontal axis indicate maximum distances defining the intervals.

subpopulation, was epigenetic similarity between individuals substantially greater than their genetic similarity. This means that the epigenetic 'kinship excess' relative to the genetic similarity baseline (null model) principally held true for plant pairs that, because of their spatial proximity, had been presumably exposed to comparable environmental conditions because of the spatial autocorrelation ordinarily shown by ecological variables (Legendre 1993). As the similarity of environmental conditions experienced by close plant pairs probably applied also to their most immediate ancestors, it is not possible with our data to assess the relative causal importance of selection-based effects (selection on heritable epialleles giving rise to adaptations in the same way as selection on genes) or detection-based effects (epialleles received by offspring depend on the environment experienced by the parents; Shea *et al.* 2011) as determinants of local epigenetic differentiation. We interpret the disproportionate (relative to the genetic null model expectations) epigenetic similarity of plant pairs growing at the closest distances, as well as the smaller size of epigenetic than genetic patches, as compelling evidence for environmentally driven intraspecific epigenetic divergence in *H. foetidus* being largely unrelated to genetic divergence. This pattern might eventually turn out to be commonplace in natural plant populations, as suggested by recent studies of Schulz *et al.* (2014) on the perennial herb *Viola elatior* and Huang *et al.* (2015) on the shrub *Rhododendron oldhamii*. In both cases, environmental factors measured locally had the greatest explanatory value to account for between-site differences in epigenetic characteristics of populations.

Taken together, results for *H. foetidus*, *V. elatior* and *R. oldhamii* suggest that, in contrast to the predominant role of IBD as the major determinant of spatial genetic structure in plants (Sexton *et al.* 2014), spatial epigenetic structure is probably to conform frequently to isolation-by-environment (IBE) models, where differences in the features of local environments are equally or more important than spatial distance as predictors of epigenetic variation. The mechanisms giving rise to epigenetic IBE patterns, however, will be different from those accounting for ordinary genetic IBE (Sexton *et al.* 2014), particularly if local environmental factors act not only as selective agents on pre-existing, heritable epigenetic variation but also as 'releasers' of new heritable epigenetic variants. Environmentally induced appearance of site-specific, heritable epigenetic variation, followed by conventional directional selection on new epigenetic variants and subsequent local epigenetic adaptation, could combine to eventually produce long-term epigenetic IBE patterns in plants. From the perspective of their adaptive potential, such epigenetic IBE patterns could complement and/or expand ordinary genetic IBD patterns commonly found in wild plant populations. The framework outlined in Fig. 2 could be easily expanded to account for situations where spatial genetic and/or epigenetic structure are determined by the combined operation of IBD and IBE.

Concluding remarks

Meirmans (2012) convincingly argued that IBD models should be routinely incorporated as null models in all studies whose goals are elucidating the spatial genetic structure and/or population differentiation of natural populations (see also Frantz *et al.* 2009). In a similar vein, albeit for different reasons, we propose in this study that genetic IBD models are valuable null models in investigations of the spatial structure of epigenetic diversity in natural plant populations. Our application to spatial genetic and epigenetic data from *H. foetidus* illustrates the heuristic value of such approach for formulating new hypotheses and improving our understanding of the factors potentially shaping epigenetic diversity in wild populations of nonmodel plants. In terms of the illustrative scheme shown in Fig. 2, we found that the spatial epigenetic structure of *H. foetidus* was consistent with individual-level IBD patterns depicted in panel 4, which provides justification for suggesting that epigenetic variation in this species has moderate-to-high transmissibility across generations and is responsive to local environmental conditions. This is supported by the high plant-to-pollen transmissibility of MSAP markers demonstrated by Herrera *et al.* (2013) for a subset of the *H. foetidus* plants considered here.

Paraphrasing and extending the famous Dobzhansky's motto (Ayala 1977), it has been suggested that 'nothing in evolution makes sense except in light of population genetics' (Lynch 2007). By the same token, understanding the actual evolutionary significance of natural epigenetic variation will require the maturation of 'population epigenetics' (Richards 2008) as a fully fledged subdiscipline focusing on the causes and implications of patterns of epigenetic variation at the individual, population and species levels (Kalisz & Purugganan 2004; Rapp & Wendel 2005; Alonso *et al.* 2015). Three of the greatest challenges confronting population epigenetics are determining the significance of natural epigenetic variation (Richards 2008; Hirsch *et al.* 2012), unravelling the relationships between genetic and epigenetic systems of inheritance (Richards 2006; Jablonka 2013; Herrera *et al.* 2014), and evaluating the long-term transgenerational stability of epigenetic diversity under natural conditions, beyond the precincts of greenhouses and growth chambers (Richards *et al.* 2010).

Application of the comparative spatial genetics and epigenetics analyses advocated in this study may contribute insights to each of these three major lines of enquiry. For example, decoupling of genetic and epigenetic spatial structure found here for *H. foetidus*, particularly the contrasting sizes of genetic and epigenetic patches, furnishes compelling evidence that genetic and epigenetic differentiation may develop independently of each other even in genetically diverse wild populations (i.e. in contrast to genetically depauperate ones created artificially for controlled experiments; see Herrera *et al.* 2014 for discussion). Contrasting epigenetic and genetic spatial structures in *H. foetidus* could be the outcome of local environmental induction of heritable epigenetic variants followed by selection over subsequent generations (Shea *et al.* 2011). This hypothesis could be tested, for example, by comparing the relative frequency of genetic and epigenetic markers exhibiting signatures of spatially divergent selection ('outlier loci'; Wenzel & Piertney 2014). The study of differences in IBD patterns between genetic and epigenetic markers may also contribute to the debated issue of the long-term transgenerational stability of epigenetic marks under natural conditions (Jablonka & Raz 2009; Hirsch *et al.* 2012; Herrera *et al.* 2014). We therefore anticipate that the modest additional effort required for georeferencing plants sampled for epigenetic studies will be amply rewarded in terms of new insights, improved hypotheses and sharper questions.

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C.M.H. and M.M. conceived and designed the study, and performed field work; P.B. performed laboratory work; C.M.H., M.M. and P.B. analysed the data; C.M.H. wrote the article.

Data accessibility

Microsatellite fingerprints and geographical coordinates of plants sampled deposited at DRYAD doi:10.5061/dryad.h8fp2. AFLP and MSAP data from Medrano *et al.* (2014) available at DRYAD doi:10.5061/dryad.fr2k8.

Supporting information

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

Fig. S1 Relationship between kinship coefficient for individual plant pairs and spatial separation for the genetic and epigenetic markers considered.

Table S1 Published information on comparative genetic and epigenetic differentiation between conspecific plant populations.