Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb

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Abstract

The ecological significance of epigenetic variation has been generally inferred from studies on model plants under artificial conditions, but the importance of epigenetic differences between individuals as a source of intraspecific diversity in natural plant populations remains essentially unknown. This study investigates the relationship between epigenetic variation and functional plant diversity by conducting epigenetic (methylation-sensitive amplified fragment length polymorphisms, MSAP) and genetic (amplified fragment length polymorphisms, AFLP) marker–trait association analyses for 20 whole-plant, leaf and regenerative functional traits in a large sample of wild-growing plants of the perennial herb *Helleborus foetidus* from ten sampling sites in south-eastern Spain. Plants differed widely in functional characteristics, and exhibited greater epigenetic than genetic diversity, as shown by per cent polymorphism of MSAP fragments (92%) or markers (69%) greatly exceeding that for AFLP ones (41%). After controlling for genetic structuring and possible cryptic relatedness, every functional trait considered exhibited a significant association with at least one AFLP or MSAP marker. A total of 27 MSAP (13.0% of total) and 12 AFLP (4.4%) markers were involved in significant associations, which explained on average 8.2% and 8.0% of trait variance, respectively. Individual MSAP markers were more likely to be associated with functional traits than AFLP markers. Between-site differences in multivariate functional diversity were directly related to variation in multilocus epigenetic diversity after multilocus genetic diversity was statistically accounted for. Results suggest that epigenetic variation can be an important source of intraspecific functional diversity in *H. foetidus*, possibly endowing this species with the capacity to exploit a broad range of ecological conditions despite its modest genetic diversity.

Keywords: community genetics, DNA methylation, epigenetic diversity, functional diversity, *Helleborus foetidus*, intraspecific variation, marker–trait association analysis

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Introduction

The crucial evolutionary importance of variation within species has been long acknowledged and thoroughly scrutinized from an endless variety of perspectives (Bowler 2005; Herrera 2009). In contrast, emphasis on the ecological consequences of intraspecific variation is a relatively recent phenomenon, largely bound to the emergence of community genetics and trait-based community ecology as two burgeoning, interrelated ecological subdisciplines (Neuhauser et al. 2003; Whitham et al. 2006; Violle et al. 2012). Intraspecific trait variability enhances the ecological breadth and distributional range of species (Sides et al. 2014) and contributes to amplify the functional diversity of plant communities, a key component of biodiversity with important implications for species coexistence and ecosystem functioning (de Bello et al. 2011; Albert et al. 2012). Genetic diversity of populations, through its effects on trait-dependent functional variability, also influences community and...
ecosystem processes (Hughes et al. 2008; Hersch-Green et al. 2011). In plant communities, genetic diversity of dominant species can be as important or more than overall species richness in shaping the diversity and abundance of associated consumers. Intraspecific genetic differences may condition the structure of herbivore communities (Whitham et al. 2006; Tack & Roslin 2011), and distinct genotypes may possess distinct functional features and play different ecological roles as if they were separate species (Crawford & Rudgers 2012).

The current community genetics framework rests on the unstated assumption that genetic variation (i.e. arising from allelic differences due to DNA sequence variants) is the only source of heritable, trait-based functional variation among conspecific genotypes (Whitham et al. 2006; Violle et al. 2012). Nevertheless, considerable evidence has accumulated in recent years showing that epigenetic variation (heritable phenotypic changes unrelated to variation in DNA sequence; Richards 2006) can also account for phenotypic differences between conspecific plants that are stably transmitted from parents to offspring (Jablonka & Raz 2009; Johannes et al. 2009; Verhoeven et al. 2010; Becker & Weigel 2012; Cortijo et al. 2014). Cytosine methylation is an important mechanism for stable epigenetic modification of DNA in plants, and heritable individual variation in phenotypic traits has been induced artificially by manipulating the pattern (distribution across specific sites in the genome) and/or level (proportion of total cytosines that are methylated) of cytosine methylation (Grant-Downton & Dickinson 2005, 2006; Jablonka & Raz 2009). Artificial modifications of patterns and/or levels of cytosine methylation can generate heritable intraspecific variation in a broad array of functional plant traits, including size, growth rate, seed size, seed production, flowering phenology, leaf size and shape, and stomatal features (Sano et al. 1990; King 1995; Finnegan et al. 1996; Fieldes & Amyot 1999; Tatra et al. 2000; Kondo et al. 2006; Amoah et al. 2012; Tricker et al. 2012).

Results of these experiments provide justification for the hypothesis that, in addition to genetic variation, epigenetic differences between individuals might be acting as a hitherto unrecognized source of intraspecific functional diversity in natural plant populations (Bossdorf et al. 2008). Additional motivation is provided by observations indicating that (i) wild plant populations harbour considerable epigenetic diversity, which often exceeds genetic diversity (Li et al. 2008; Herrera & Bazaga 2010; Lira-Medeiros et al. 2010); (ii) intraspecific variation in global DNA cytosine methylation may be associated with differences in functional traits (Alonso et al. 2014); and (iii) epigenetic diversity may enhance the colonizing ability, productivity, recruitment and stability of plant populations (Richards et al. 2012; Latzel et al. 2013; Herrera et al. 2014). All these findings point to a role of epigenetic variation as a source of intraspecific functional diversity.

With few exceptions, studies on the ecological significance of epigenetic variation have been conducted on model plants under artificial conditions, and the hypothesis relating epigenetic variation to intraspecific functional diversity remains largely unexplored in natural populations (but see Richards et al. 2012; Alonso et al. 2014; Schulz et al. 2014). In this study, we undertake epigenetic marker–trait association analyses on a large sample of wild-growing plants of the herb Helleborus foetidus to test the predicted relationship between epigenetic variation and functional diversity in this species. Individual epigenetic differences may or may not be independent of individual genetic differences (i.e. DNA sequence-based) (Richards 2006; Herrera & Bazaga 2010; Herrera et al. 2014), hence genetic markers were also included in the association analyses. This will allow, on one side, to evaluate the association between epigenetic variation and trait variation while controlling for the effects of genetic heterogeneity and, on the other, to compare the quantitative importance of genetic and epigenetic variation as predictors of intraspecific functional diversity. Epigenetic and genetic marker–trait associations will be sought for a total of 20 whole-plant, leaf and regenerative traits (listed in Table 1) measured on individual wild-growing plants. All plant features considered here qualify as functional traits, as they may directly or indirectly affect the fitness or the environment of individuals (Pérez-Harguindeguy et al. 2013).

Materials and methods

Study plant and field sampling

Helleborus foetidus L. (Ranunculaceae) is a perennial, evergreen herb widely distributed in western and south-western Europe, where it can be found from sea level to 2100 m elevation in a broad variety of habitat types ranging from open scrub to conifer and broad-leaved forests (Mathew 1989). Adult plants generally consist of vegetative and reproductive ramets arising from a small, weakly developed rhizome (Werner & Ebel 1994). After several seasons of vegetative growth, each vegetative ramet produces a single terminal inflorescence and dies following fruit maturation and seed shedding. In our study area (see below), flowering mostly takes place during February–April. Each inflorescence produces 25–75 flowers over its 1.5–2.5 months flowering period, and bumble bees are the main pollinators. Fruit maturation and seed shedding occur in June-early July.
Field sampling for this study was conducted during 2012 and 2013 in the Sierra de Cazorla, a well-preserved mountain area in Jaén province, south-eastern Spain. *Helleborus foetidus* is widely distributed there over a broad range of elevations and habitat types. Plants were sampled at ten locations, chosen to encompass the entire ecological range of the species in the region (Appendix S1, Supporting information). Sites included the three localities studied by Herrera et al. (2013, 2014). At each locality, 20 widely spaced, inflorescence-bearing plants were randomly selected during February–May 2012, marked with permanent tags and georeferenced using a GPS receiver. Sampled plants were distributed over roughly similar areas at all sites (Appendix S1, Supporting information). Elevational differences between sites resulted in phenological variation. To avoid developmental variation in DNA methylation confounding individual differences in methylation patterns, leaf samples for molecular analyses were collected at each site during the local flowering peak. Young expanding leaves were collected from each plant, placed in paper envelopes and dried immediately at ambient temperature in sealed containers with silica gel. This material was used for genetic and epigenetic fingerprinting of plants and also for leaf carbon isotope ratio measurements (see below). The following traits were recorded for every plant at the time of leaf collection in 2012: number of vegetative and reproductive ramets, diameter of inflorescence at the base, number of flowers per inflorescence and age in years of flowering ramets as determined from counts of annual marks left on stems by the abruptly shrinking late-season internodes (Werner & Ebel 1994). Length of floral perianth (‘corolla length’ in Herrera et al. 2002) was measured on two randomly chosen flowers from each plant, and the mean used as an estimate of flower size. The number of follicles eventually developing into ripe fruits was determined for each plant shortly before fruit maturation. For each plant, a sample of ripe seeds were collected from 2 to 9 different fruits (range = 10–15 seeds per plant), weighed individually to the nearest 0.1 mg, and a mean seed mass value obtained.

<table>
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<tr>
<th>Trait class</th>
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<th>Trait code</th>
<th>Measurement unit</th>
<th>Number of plants measured</th>
<th>Range</th>
<th>CV* (%)</th>
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<td>Whole plant</td>
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<td>Number</td>
<td>Number</td>
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<td></td>
<td>201</td>
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*CV = coefficient of variation, ratio of standard deviation to the mean expressed as percentage.

†Stomatal index = 100 * (stomatal density)/(stomatal density + epidermal cell density).
leaves of the same plant (Mathew 1989; Werner & Ebel 1994). To account for this variation and obtain figures that are comparable between plants, foliar trait measurements were taken on the central leaflet of leaves from different parts of the plant, sampled using a stratified scheme. The two longest vegetative ramets were selected from each plant. From each ramet, the central leaflets of two nonadjacent, undamaged, mature leaves were collected, labelled, and kept into sealed plastic bags in a cooler until processed in the laboratory.

**Laboratory methods**

Genetic and epigenetic characteristics of *Helleborus foetidus* plants sampled were assessed by fingerprinting them using amplified fragment length polymorphism (AFLP; Weising et al. 2005; Meudt & Clarke 2007) and methylation-sensitive amplified polymorphism (MSAP; Schulz et al. 2013; Fulneček & Kovárík 2014) techniques. Total genomic DNA was extracted from dry leaf samples using Qiagen DNAeasy Plant Mini Kit and the manufacturer protocol. AFLP and MSAP analyses were conducted on the same DNA extracts. The AFLP analysis was performed using standard protocols involving the use of fluorescent dye-labelled selective primers (Weising et al. 2005). Restriction–ligation was conducted using *Pst*I/*Mse*I endonuclease mixture and double-stranded adaptors. After an initial screening of primer pair combinations, a total of eight *Pst*I + 2/*Mse*I + 3 primer pairs were chosen that provided reliable, consistently scorable results, and each plant was fingerprinted using these combinations (Table S1, Supporting Information). Fragment separation and detection was made using an ABI PRISM 3130xl DNA sequencer, and the presence or absence of each AFLP fragment in each individual plant was scored manually by visualizing electropherograms with *Genemapper* 3.7 software. Only fragments ≥150 base pairs in size were considered to reduce possible biases arising from size homoplasy (Vekemans et al. 2002; Caballero et al. 2008). AFLP genotyping error rates were determined for each primer combination by running repeated, independent analyses for 27 (combinations 1–4, Table S1, Supporting information; 13.5% of total plants) or 20 plants (combinations 5–8, Table S1, Supporting information; 10% of total) and estimated as the ratio of the number of discordant scores in the two analyses (all plants and markers combined) to the product of the number of plants by the number of scored markers (Herrera & Bazaga 2009). Average genotyping error rate (±SE) for the eight AFLP primer combinations used was 1.7 ± 0.3% (Table S1, Supporting information).

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, commonly *Eco*RI or Msel. Msel was used here because of better repeatability of results (see also Verhoeven et al. 2010; Herrera et al. 2013; for earlier MSAP implementations based on Msel). The recognition sequence of Msel (5’-TTAA-3’) is shorter than that of *Eco*RI (5’-GTTAAC-3’), which leads to more frequent cuts and hence an expected reduction in the incidence of blind internal 5’-CCGG-3’ targeted sites (Fulneček & Kovárík 2014). More importantly, Msel cleavage site does not contain any cytosine residue, which produces a ‘methylation-indifferent cutting’ independent of cytosine presence and methylation status. *Hpa*II and *Msp*I are isoschizomers that recognize the same tetranucleotide 5’-CCGG but have differential sensitivity to methylation at the inner or outer cytosine. Differences in the products obtained with *Hpa*II and *Msp*I thus reflect different methylation states at the cytosines of the CCGG sites recognized by *Hpa*II or *Msp*I cleavage sites (see Schulz et al. 2013; Fulneček & Kovárík 2014, for references and further details). MSAP assays were conducted on DNA samples from all *H. foetidus* plants sampled using four *Hpa*II-*Msp*I + 2/*Mse*I + 3 primer combinations (Table S1, Supporting information). Fragment separation and detection was made using an ABI PRISM 3130xl DNA sequencer, and the presence or absence of *Hpa*II/*Mse*I and *Msp*I/*Mse*I fragments in each sample was scored manually by visualizing electropherograms with *Genemapper* 3.7 software. MSAP genotyping error rates were estimated for each primer combination by running repeated *Hpa*II/*Mse*I and *Msp*I/*Mse*I analyses for 17 plants (8.5% of total) and computed as the ratio of the number of discordant scores in the two analyses (all plants and markers, and the two enzyme pairs, combined) to twice the product of the number of plants by the number of scored markers. Mean genotyping error rate (±SE) for the four MSAP primer combinations used was 3.7 ± 0.5% (Table S1, Supporting information).

Leaf carbon isotope ratios were measured following standard protocols (Pérez-Harguindeguy et al. 2013) on the same leaf material used for DNA extraction. Weighed powdered leaf samples were placed into tin capsules and combusted at 1020 °C using continuous flow isotope ratio mass spectrometry by means of a Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CON- FLO IV interface (Thermo Fisher Scientific, Bremen, Germany). Results were expressed relative to the Pee Dee Belemnite standard as δ¹³C in per mil units (‰) (Pérez-Harguindeguy et al. 2013).

All collected leaflets from marked plants were mounted individually on paper sheets while still in fresh condition, digitally scanned and then desiccated.
After calibration of digital images, total area, maximum length and maximum width of each leaflet were measured using *SIGMA SCAN PRO* (version 5.0; Systat Software Inc., San Jose, CA, USA). Dried leaflets were weighed on an analytical balance to the nearest 0.01 mg, and specific area calculated as the area to dry mass ratio (Pérez-Harguindeguy et al. 2013). The impression approach (e.g. Peterson et al. 2012) was used to measure stomatal traits. Transparent impressions of the widest portion of the abaxial surface of each leaflet were created using clear nail polish and mounted on microscope slides. Stomatal density (number of stomata per mm²) was estimated for five fields of view widely spaced across each impression at 400 × magnification (field of view = 0.283 mm²). Two photomicrographs were taken from haphazardly selected, nonoverlapping areas of each impression at 100 × magnification using Nomarski differential interference contrast optics. Counts of stomata and epidermal cells were performed on two nonadjacent quadrats of a 0.0625 mm² grid overimposed on each image, and the area independent stomatal index (SI) was calculated as SI = [s/(e + s)] × 100, where s is the number of stomata and e is the number of epidermal cells (Salisbury 1927). Guard cells were not included in the number of epidermal cells. Stomatal length, defined as the distance between the junctions of guard cells at opposite ends of stomata, was measured on 20 randomly chosen, open stomata per impression (10 per photomicrograph). Replicate measurements of leaflet and stomatal traits from the same plant were averaged to obtain single values per trait and individual.

**Data analysis**

Two presence–absence matrices for MSAP fragments were obtained with the four *HpaII-MseI* and *MspI-MseI* primer combination pairs (Table S1, Supporting information). Different workflows (MSAP ‘scoring’ methods) have been proposed to obtain from these raw data the information (Peterson et al. 2012) was used to measure stomatal traits. Transparent impressions of the widest portion of the abaxial surface of each leaflet were created using clear nail polish and mounted on microscope slides. Stomatal density (number of stomata per mm²) was estimated for five fields of view widely spaced across each impression at 400 × magnification (field of view = 0.283 mm²). Two photomicrographs were taken from haphazardly selected, nonoverlapping areas of each impression at 100 × magnification using Nomarski differential interference contrast optics. Counts of stomata and epidermal cells were performed on two nonadjacent quadrats of a 0.0625 mm² grid overimposed on each image, and the area independent stomatal index (SI) was calculated as SI = [s/(e + s)] × 100, where s is the number of stomata and e is the number of epidermal cells (Salisbury 1927). Guard cells were not included in the number of epidermal cells. Stomatal length, defined as the distance between the junctions of guard cells at opposite ends of stomata, was measured on 20 randomly chosen, open stomata per impression (10 per photomicrograph). Replicate measurements of leaflet and stomatal traits from the same plant were averaged to obtain single values per trait and individual.

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To determine whether, and the extent to which, differences between individual *H. foetidus* plants in functional features were associated with their genetic and/or epigenetic characteristics, we looked for AFLP and MSAP markers significantly associated with the 20 functional traits considered (Table 1). For each trait, separate linear mixed-effects models were fit for each AFLP and MSAP marker using REML estimation. Computations were performed using the lme function from the nlme package for the R environment (R Development Core Team 2012). In each model, the functional trait was the dependent variable and marker presence–absence the single fixed-effect, two-level factor. Application of Bayesian clustering to the AFLP fingerprint data revealed that plants sampled were genetically structured, falling into one of two genetically distinct clusters (Appendix S2, Supporting information; clusters comprised the six northernmost and four southernmost sampling sites, respectively). Genetic stratification and possible relatedness of sampled plants could produce spurious marker–trait associations (Price et al. 2010; Siillanpää 2011). To correct, at least in part, for these possible confounding effects, genetic cluster and sampling site nested within genetic cluster, were incorporated as random effects in the mixed models (Price et al. 2010). *P*-values for the effect of marker presence–absence on a given trait were used to identify significant associations. Given the large number of models fit for every trait, Storey & Tibshirani’s (2003) *q*-value method was applied to estimate false discovery rates. Using the qvalue package (Storey & Tibshirani 2003), we calculated for every trait the set of *q*-values for all marker–trait models fitted, and found the largest *q*-value leading to an expectation of less than one falsely significant model [i.e. \(q\text{-value} \times \text{(number of models accepted as significant)} < 1\)].

In addition to identifying AFLP and MSAP markers significantly associated with the functional traits considered, we were also interested in quantifying the explanatory value of these significant markers as predictors of intraspecific functional diversity in *H. foetidus*. © 2014 John Wiley & Sons Ltd
Whenever applicable, three separate linear mixed-effects models were fit for each trait, which had trait value as the response variable, and included all significant AFLP markers, all significant MSAP markers and all significant AFLP + MSAP markers, respectively, as fixed effects (predictors). As performed in models fitted to identify significant markers, genetic cluster and sampling site nested within genetic cluster were also included as random effects. The marginal $R^2$ for each model, which represents the variance explained by fixed factors alone (Nakagawa & Schielzeth 2013), was used to evaluate the explanatory value of significant epigenetic and genetic markers, taken separately and in combination.

The within- and between-site components of sample-wide variance in functional traits were computed by fitting random effects models to the data using REML estimation, and confidence intervals of variance estimates obtained by bootstrapping. Between-site differences in functional diversity of *H. foetidus* plants, and their relationship to local genetic and epigenetic diversity, were tested using a multivariate version of Van Valen’s test for homogeneity of variances, which tests the mean absolute deviation rather than the variance (Van Valen 2005). Principal coordinates analyses of pairwise distance matrices were used to obtain plant coordinates on each of five reduced-dimensionality spaces, defined, respectively, by functional traits and the scores for significant AFLP markers, nonsignificant AFLP markers, significant MSAP markers and nonsignificant MSAP markers. Individual distances to the respective group centroids were then computed on each of these spaces. Site means were used as measurements of multivariate dispersion, and significance of between-site differences was tested using an analysis of variance approach. Computations were performed with function betadisper of the vegan package. Relationships across sites between multivariate functional diversity, on one side, and multilocus genetic and epigenetic diversity of significant and nonsignificant markers, on the other, were tested by fitting ordinary linear models to site means.

**Results**

**Genetic and epigenetic diversity**

All *Helleborus foetidus* plants sampled were fingerprinted using 674 AFLP and 155 MSAP fragments (Table S1, Supporting information). After scoring, the vast majority of MSAP fragments (91.6%) led to one or more distinct MSAP marker types, and a combined total of 296 $u$-, $h$- and $m$-type markers were obtained (Table S1, Supporting information). Only the 270 AFLP and 207 MSAP (64 $u$-type, 99 $h$-type, 44 $m$-type) polymorphic markers (at least 2% of samples showing a variant score) were retained for study (Table S1, Supporting information). Per cent marker polymorphism per primer combination was considerably higher for MSAP (68.6 ± 3.2% polymorphism, $N = 12$, $u$-, $m$- and $h$-type markers combined) than for AFLP markers (41.1 ± 3.4% polymorphism, $N = 8$), the difference being statistically significant (chi-squared = 15.25, d.f. = 1, $P < 0.0001$; Kruskal–Wallis rank sum test).

**Intraspecific variation in functional traits**

Plants of *Helleborus foetidus* differed widely in most of the 20 functional traits considered, as denoted by broad ranges and large coefficients of variation (CV = 100 x standard deviation/mean) of individual values (Table 1). There was a trend for increasing individual variability from leaf (mean CV ± SE = 23 ± 5%; means are reported ± SE throughout this study) through regenerative (44 ± 15%) to whole-plant (53 ± 7%) functional traits, but differences between trait classes did not reach statistical significance (chi-squared = 4.07, d.f. = 2, $P = 0.13$; Kruskal–Wallis rank sum test). Flower length (CV = 5.8%), leaf carbon isotope ratio (6.0%), and stomata length (6.6%) were the least variable traits. On the opposite extreme, number of follicles ripened (CV = 102.2%), number of flowers produced (82.8%) and number of vegetative ramets (66.6%) were the most variable traits (Table 1).

Although means for all traits considered differed significantly among sampling sites (results not shown), most variation occurred within sites. On average, 70.7 ± 3.6% ($N = 20$ traits) of sample-wide variance was due to individual variation within sites. For 15 traits, within-site variance component was greater than 50% and the 95% confidence interval did not include that value, thus denoting that within-site variance significantly exceeded between-site variance (Fig. 1). Four leaf-related traits (width, length, area and specific area) exhibited the smallest proportions of within-site functional variance (range = 39.4–52.6%; Fig. 1). On the opposite extreme, three regenerative traits were among those exhibiting the highest levels of within-site variance (number of flowers, number of seeds, number of follicles; range = 85.3–93.5%; Fig. 1).

**Marker–trait associations**

A total of 477 polymorphic markers (270 AFLP, 207 MSAP) were tested for significant associations with the 20 functional traits considered, and 50 instances were found (Table 2). Every trait had at least one significantly associated MSAP or AFLP marker.

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Instances of significant AFLP and MSAP marker–trait associations were nonrandomly distributed across traits ($P = 0.00011$, Fisher’s exact probability test). Individual traits tended to be associated exclusively with either MSAP

Table 2 Number of epigenetic (MSAP) and genetic (AFLP) markers significantly associated with functional traits in the sample of *Helleborus foetidus* plants studied (see Table S2, Supporting information for marker identification and further details), and proportion of sample-wide variance explained, as assessed with the marginal $R^2$ of the corresponding mixed-effects models

<table>
<thead>
<tr>
<th>Functional trait</th>
<th>Significantly associated markers</th>
<th>Marginal $R^2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSAP</td>
<td>AFLP</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Vegetative ramets</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Reproductive ramets</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total ramets†</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Age of flowering ramets</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Leaflet length</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Leaflet width</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Leaflet area</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Leaflet dry mass</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Specific leaf area</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Stomata length</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stomatal density</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Stomatal index</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Leaf carbon isotope ratio</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Inflorescence basal diameter</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Flower length</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Follicles per flower</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total flowers produced†</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total follicles ripened†</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total seeds produced†</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Seed mass</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

*For each functional trait, all markers deemed significant had associated $P$-values ≤ than the trait-specific threshold shown, and taken together, the expected number of false positives was <1.

†Data were log$_{10}$-transformed for the analyses.

(mean ± SE = 2.5 ± 0.3 associated markers per trait, or 0.52% of markers assayed per trait; Table 2). Instances of significant AFLP and MSAP marker–trait associations were nonrandomly distributed across traits ($P = 0.00011$, Fisher’s exact probability test). Individual traits tended to be associated exclusively with either MSAP

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(e.g. vegetative ramets, stomatal density, leaf carbon isotope ratio, follicles per flower) or AFLP (e.g. reproductive ramets, specific leaf area, stomatal index) markers more often than would be expected by chance (Table 2).

The 50 instances of significant marker–trait associations involved 20 distinct MSAP fragments (12.9% of total), 27 distinct MSAP markers (13.0% of total) and 12 distinct AFLP markers (4.4% of total) (Table S2, Supporting information). On a per-fragment or per-marker basis, therefore, the probability of MSAP markers being significantly associated with some functional trait roughly triplicated that for AFLP markers, the difference being statistically significant ($P = 0.0011$, Fisher’s exact probability test).

**Explanatory value of significant genetic and epigenetic markers**

After statistically controlling for genetic background heterogeneity and possible cryptic relatedness of plants from the same site, significant AFLP and MSAP markers combined explained on average 9.8 ± 1.0% (marginal $R^2$ from mixed models; range = 2.2–17.9%, $N = 20$ traits) of trait variance, while significant AFLP and MSAP markers considered separately explained on average 8.0 ± 1.4% (range = 2.2–16.8%) and 8.2 ± 0.9% (range = 3.3–17.9%) of trait variance, respectively (Table 2). Individual traits differed widely, however, in the relative explanatory value of significantly associated AFLP and MSAP markers, as estimated by the marginal $R^2$ of the corresponding models (Fig. 2). Certain traits had a considerable amount of variance explained by either MSAP (e.g. vegetative ramets, stomatal density, leaf carbon isotope ratio, flower length) or AFLP markers alone (e.g. stomatal index, reproductive ramets, inflorescence basal diameter), while in other cases MSAP and AFLP markers had roughly similar explanatory values (e.g. leaflet dry mass, stomata length, leaflet area) (Fig. 2). There was no discernible relationship between functional trait class and the explanatory value of significant AFLP and MSAP markers.

**Functional, genetic and epigenetic diversity across sampling sites**

Multivariate functional diversity of locally coexisting *Helleborus foetidus* plants, estimated by the mean distance of individuals to the corresponding site centroid in the reduced-dimensionality functional space, differed significantly between sampling localities ($\chi^2 = 47.6$, d.f. = 9, $P < 0.0001$; Kruskal–Wallis rank sum test). Differences between sites in functional diversity were positively and significantly related to variation in local multilocus diversity of significant genetic (AFLP) and epigenetic (MSAP) markers, but unrelated to differences in local multilocus diversity of nonsignificant markers of any type (Table 3). Between-site differences in local epigenetic and genetic diversity accounted for 83% of variation in local functional diversity (Table 3), which suggests a major predictive value of variations in genetic and epigenetic diversity as determinants of population differences in functional diversity.

![Fig. 2 Proportion of variance of the different functional traits considered that was explained by the respective sets of significantly associated epigenetic (MSAP) and genetic (AFLP) markers (see Tables 2 and S2, Supporting information), as estimated by marginal $R^2$ from mixed-effects models. Trait codes and classes as in Table 1.](image)
Intraspecific variation in the wild populations of *Helleborus foetidus* sampled was extensive, implicating the vast majority of the 20 traits considered and occurred predominantly at the within-population level. These results corroborate and extend those of recent studies which, although generally based on limited sets of traits, have also documented the quantitative importance of intraspecific diversity in wild plant populations (Iannetta *et al.* 2007; Boucher *et al.* 2013; Mitchell & Bakker 2014). The magnitude of intraspecific variation exhibited by *H. foetidus* is well illustrated by comparing, for example, the ranges of individual values for specific leaf area (a moderately variable trait, range = 8–26 mm²/mg, Table 1) and leaf carbon isotope ratio (a relatively constant trait, range = –30 to –22‰, Table 1) with the corresponding interspecific ranges reported for large multispecies samples worldwide (1–69 mm²/mg, 2370 species from Wright *et al.* 2004; –33 to –23‰; 146 species from Körner *et al.* 1988). For these two traits, the extent of intraspecific variation over the relatively small spatial scale of this study was thus large enough to encompass a major fraction of the corresponding ranges of interspecific variation worldwide. All traits examined here are expected to be consequential for the plants’ fitness or their immediate environment, as discussed below. Consequently, the finding that intraspecific variation can sometimes be nearly as large as interspecific differences stresses once more the importance of considering intraspecific variation in studies of community and ecosystem function (de Bello *et al.* 2011; Violle *et al.* 2012; Mitchell & Bakker 2014; Sides *et al.* 2014).

Traits considered in this study can have direct or indirect functional consequences for plants, and their variation at the regional and within-population levels will be associated with intraspecific functional diversity. Among leaf traits, for instance, carbon isotope composition reflects intrinsic water use efficiency; specific leaf area is directly related to mass-based photosynthetic rate; variations in leaf area and linear dimensions are related to lifespan and thermal balance; and size and density of stomata are key factors in water economy, gas exchange and net carbon assimilation (Ackerly & Reich 1999; Westoby *et al.* 2002; Wright *et al.* 2004; Pérez-Harguindeguy *et al.* 2013). Variation in life history (e.g. age of flowering ramets, plant size) and fecundity-related traits (e.g. fruit and seed production, seed size) will directly influence the turnover, recruitment, age structure, productivity and persistence of populations (Harper 1977). Variations in size of inflorescences and individual flowers can influence fecundity through effects on pollinator attractiveness and pollination success, because seed production by winter-flowering *H. foetidus* is often pollen-limited in the study region (Herrera 2002).

After statistically controlling for the potential confounding effects of genetic structuring and possible relatedness of sampled plants, all functional traits considered exhibited a significant association with at least one AFLP or MSAP marker. Interpretation of these results is subject to the usual caveat that significant marker–trait associations provide only indicative evidence and are not by themselves a conclusive proof of causality (e.g. Platt *et al.* 2010). Keeping this in mind, circumstantial evidence does support the interpretation that significant marker–trait associations found here can stem from the markers involved being linked to genomic regions directly controlling the associated traits in causative ways. In plant genomes, AFLP markers are often positioned within gene sequences or linked to QTLs of known phenotypic effects, including some of the traits considered here such as leaf area, specific leaf area, carbon isotope discrimination and seed size (Teulat *et al.* 2002; Scafli *et al.* 2004; Caballero *et al.* 2013). Although they have been investigated much less often, there are also clear indications that MSAP markers can be stably associated across generations and environments with genes or QTL of diverse

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### Table 3 Relationship across the 10 sampling sites between multivariate functional diversity of *Helleborus foetidus* plants and multilocus epigenetic and genetic diversity, tested separately for subsets of markers significantly and nonsignificantly related to functional traits (Tables 2 and S2, Supporting information)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Parameter estimate ± SE</th>
<th>$F_{1,7}$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Significant markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model fit: $F_{2,7} = 23.79$, adjusted $R^2 = 0.83$, $P = 0.0007$</td>
<td>Epigenetic diversity</td>
<td>0.267 ± 0.105</td>
<td>6.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genetic diversity</td>
<td>0.898 ± 0.159</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nonsignificant markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model fit: $F_{2,7} = 0.76$, adjusted $R^2 = 0.02$, $P = 0.5$</td>
<td>Epigenetic diversity</td>
<td>0.141 ± 0.133</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genetic diversity</td>
<td>0.014 ± 0.050</td>
</tr>
</tbody>
</table>
phenotypic effects, including some of the traits considered here (Long et al. 2011; Tricker et al. 2012). For instance, transgenerationally heritable variations in stomatal development and water use efficiency are due to stable alterations in the methylation status of specific genes or genomic sites (Wang et al. 2011; Tricker et al. 2012). Regardless of the underlying mechanistic basis, however, marker–trait associations revealed by this study indicate that, in addition to genetic variation, individual differences in the methylation status of specific zones in the genome also predict intraspecific variation in life history, fecundity and leaf traits of functional significance.

Results of this study support the hypothesis that, apart from the usually acknowledged effects of genetic diversity, natural epigenetic variation can also contribute to enhance intraspecific functional diversity in wild plant populations (Bossdorf et al. 2008; Richards et al. 2012; Alonso et al. 2014). In addition to the marker–trait relationships identified at the regional scale, another result supporting this hypothesis was the finding that between-site variation in multivariate functional diversity of local *H. foetidus* populations was directly related to differences in multilocus epigenetic diversity after multilocus genetic diversity was statistically accounted for. Given the low statistical power of the test due to the limited number of sites sampled, this result suggests that the effect of local epigenetic diversity on local functional diversity should be quantitatively important. The importance of epigenetic variation in explaining intraspecific functional diversity varied widely among traits. Individual traits tended to be predominantly associated with either MSAP or AFLP markers alone, which led to intraspecific trait variance being mostly explained by either epigenetic or genetic variation, respectively (Fig. 2). Although it is not possible at present to propose a mechanistic basis for these results, further evidence on the independence (orthogonality) of AFLP and MSAP markers as predictors of functional traits is furnished by the almost perfect additivity of AFLP and MSAP markers as predictors of functional traits. Individual traits had roughly similar explanatory value (marginal $R^2 = 8.0\%$ and $8.2\%$, respectively), which would perhaps suggest quantitatively similar roles of genetic and epigenetic variation as predictors of intraspecific functional diversity in *H. foetidus*. Our results, however, may slightly underestimate the predictive value of epigenetic relative to genetic variation, because the number of polymorphic AFLP markers assayed (270) exceeded that of variable MSAP fragments (142) and markers (207). The per-marker probability of being significantly associated with some trait was considerably higher for MSAP (0.130) than for AFLP markers (0.044). Had we assayed an equivalent number of MSAP and AFLP markers, the number of MSAP markers significantly associated with traits, and hence their overall predictive value, would possibly have been higher.

Studies on model plants under artificial conditions have firmly established that DNA methylation patterns at specific genomic sites most often are stably transmitted from parents to offspring (Johannes et al. 2009; Cortijo et al. 2014; Li et al. 2014), but comparatively little is known on the transgenerational transmissibility of methylation patterns in wild plant populations. Prior investigations conducted at three of the *H. foetidus* populations studied here found that, averaged over plants, 84% of MSAP markers had their methylation status unchanged from plant to pollen (i.e. sporophyte to gametophyte stages), thus demonstrating extensive postmeiotic epigenetic stability in this species (Herrera et al. 2013, 2014). In addition, multilocus epigenetic differentiation between *H. foetidus* populations was preserved from the sporophyte to the gametophyte stage despite a certain amount of epigenetic reprogramming during gametogenesis (Herrera et al. 2013). Although the set of MSAP markers considered in the present study only partly overlap those used in these prior investigations on transmissibility, some shared markers found here significantly associated with functional traits had transmissibilities $\geq 90\%$ (C. M. Herrera and M. Medrano, Unpublished). Methylation patterns could be reprogrammed during early embryo development, and methylation patterns of pollen grains might differ from methylations of sperm cells (see Herrera et al. 2013, for review and discussion), yet these earlier results lead us to tentatively suggest that, insofar as marker–trait associations found here reflect causal relationships, population and individual variability of epigenetically influenced traits will stably persist across a number of generations in *H. foetidus* in our study region. In this way, epigenetically mediated functional variability would propagate transgenerationally in a way similar to variability in genetically influenced traits. Long-term field experiments are currently underway to test this prediction. It must be stressed, however, that even if individual and population differences in methylation patterns were quite imperfectly transmitted across generations, the relationship between epigenetic variation and intraspecific functional diversity documented in this study would still retain its ecologically relevance, as discussed in the next paragraph.

Intraspecific trait variability may enhance the ecological breadth and distributional range of species, as illustrated by Sides et al. (2014) for a set of 21 mountain plants whose elevational ranges were directly related to

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variability in specific leaf area, one of the traits considered here. Consistent with this finding are the extensive variability in functional traits exhibited by *H. foetidus* in our study region, and the broad range of elevations and habitat types inhabited by the species there and elsewhere (Mathew 1989), which presumably denotes an ability to exploit wide environmental gradients. Our finding that intraspecific functional variability at the regional (all sites pooled) and local (within sites) levels was significantly predicted by epigenetic variation points to an effective role of epigenetic diversity in allowing *H. foetidus* to exploit contrasting environments. In addition to the higher per-marker probability of MSAP markers of predicting functional variation relative to AFLP ones discussed above, additional evidence likewise suggests that epigenetic variation might be at least as important as genetic variation in explaining the broad ecological niche and high functional diversity of *H. foetidus*. Per cent polymorphism of MSAP fragments and markers greatly exceeded that of AFLP markers, denoting considerably greater epigenetic than genetic diversity as found in other species (see references in Introduction). In addition, *H. foetidus* has characteristically low levels of genetic variation, as revealed not only by the modest polymorphism of AFLP markers shown here (41%), but also by the low polymorphisms obtained in screenings of other genetic markers using samples from broad geographical areas (50% polymorphism for 26 nuclear microsatellites, 7% polymorphism for 13 allozyme loci; Consortium MERPD et al. 2013; M. Medrano, Unpublished). We therefore interpret results of this study as an indication that, by contributing significantly to the broad intraspecific functional diversity of *H. foetidus*, epigenetic variation possibly allows this species to exploit a broad range of ecological conditions despite its modest genetic diversity.

**Acknowledgements**

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**References**


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Hersch-Green EI, Turley NE, Johnson MTJ (2011) Community genetics: what have we accomplished and where should we be going? *Philosophical Transactions of the Royal Society B-Biological Sciences*, 366, 1453–1460.


C.M.H. and M.M. conceived and designed the experiments; C.M.H., M.M. and P.B. performed the experiments; C.M.H., M.M. and P.B. analysed the data; and C.M.H. and M.M. wrote the manuscript.

Data accessibility

AFLP, MSAP and functional trait data used in this study deposited at DRYAD: doi:10.5061/dryad.fr2k8.

Supporting information

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

Appendix S1 Location and characteristics of sampling sites.

Appendix S2 Genetic structure of Helleborus foetidus plants sampled for the study.

Table S1 Primer combinations, scoring errors, number of markers and polymorphism levels for AFLP and MSAP analyses.

Table S2 AFLP and MSAP markers significantly associated with functional traits.