Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (Aquifoliaceae) trees

CARLOS M. HERRERA* and PILAR BAZAGA

Estación Biológica de Doñana, CSIC, Avenida Américo Vespucio s/n, E-41092 Sevilla, Spain

Phenotypic plasticity is central to the persistence of populations and a key element in the evolution of species and ecological interactions, but its mechanistic basis is poorly understood. This article examines the hypothesis that epigenetic variation caused by changes in DNA methylation are related to phenotypic plasticity in a heterophyllous tree producing two contrasting leaf types. The relationship between mammalian browsing and the production of prickly leaves was studied in a population of *Ilex aquifolium* (Aquifoliaceae). DNA methylation profiles of contiguous prickly and nonprickly leaves on heterophyllous branchlets were compared using a methylation-sensitive amplified polymorphism (MSAP) method. Browsing and the production of prickly leaves were correlated across trees. Within heterophyllous branchlets, pairs of contiguous prickly and nonprickly leaves differed in genome-wide DNA methylation. The mean per-marker probability of methylation declined significantly from nonprickly to prickly leaves. Methylation differences between leaf types did not occur randomly across the genome, but affected predominantly certain specific markers. The results of this study, although correlative in nature, support the emerging three-way link between herbivory, phenotypic plasticity and epigenetic changes in plants, and also contribute to the crystallization of the consensus that epigenetic variation can complement genetic variation as a source of phenotypic variation in natural plant populations.

ADDITIONAL KEYWORDS: herbivory – heterophylly – induced defences – leaf dimorphism – spinescence – subindividual variation.

INTRODUCTION

The ability of individual genotypes to produce different phenotypes in response to variations in the environment, or phenotypic plasticity, is central to the persistence of populations and a key element in the evolution of species and ecological interactions (Schlichting & Pigliucci, 1998; Agrawal, 2001; DeWitt & Scheiner, 2004; Herrera, 2009; Wund, 2012). Although all organisms exhibit some degree of phenotypic plasticity, it is among higher plants that the capacity of genotypes to produce alternative phenotypes in response to the environment is most conspicuous and has been most thoroughly investigated (reviewed by, for example, Schlichting, 1986; Sultan, 1987; Schlichting & Pigliucci, 1998; Núñez-Farfán & Schlichting, 2001; Herrera, 2009). The modular organization of higher plants, entailing the reiteration of homologous organs (e.g. leaves, flowers) by the same genotype, leads to phenotypic plasticity being most often expressed at a subindividual level in the form of variation in traits of reiterated organs (de Kroon *et al.*, 2005; Herrera, 2009). Continuous variation among homologous organs produced by the same plant is universal and frequently exceeds between-individual variation, although it is discrete variation that has traditionally furnished the most eye-catching illustrations of the ability of single genotypes to produce contrasting phenotypes (Herrera, 2009). One of the most celebrated examples of phenotypic

*Corresponding author. E-mail: herrera@ebd.csic.es

plasticity in plants is heterophylly, which involves 'the concurrent variation in leaf form within a single plant' (Zotz, Wilhelm & Becker, 2011). Heterophyllyis particularly frequent in certain ecological scenarios (e.g. aquatic habitats and oceanic islands; Sculthorpe, 1967; Friedmann & Cadet, 1976; Givnish *et al*., 1994; Wells & Pigliucci, 2000), but it is widespread worldwide, and its study has furnished some of the clearest examples of the functional significance and adaptive value of plant phenotypic plasticity (Cook & Johnson, 1968; Winn, 1996, 1999; Wells & Pigliucci, 2000; Minorsky, 2003).

Although environmental and life history correlates of phenotypic plasticity are reasonably well understood theoretically (e.g. Pigliucci, 2001; Sultan & Spencer, 2002; DeWitt & Scheiner, 2004), its mechanistic basis is poorly known, largely because of limitations inherent to the statistically oriented, 'black box' approaches typically adopted by studies of phenotypic responses to variable environments (Scheiner, 1993; Pigliucci, 1996). Recent molecular tools, however, have opened up new opportunities for unravelling the mechanisms that allow individual genotypes to cope with variable environments (Pigliucci, 2001; Aubin-Horth & Renn, 2009). There have been recent suggestions, for example, that changes in DNA methylation independent of sequence variation may underlie phenotypic plasticity, but this possibility remains essentially untested (Bossdorf, Richards & Pigliucci, 2008; Bossdorf *et al*., 2010; Richards, Bossdorf & Pigliucci, 2010; Richards, 2011; Herrera, Pozo & Bazaga, 2012). The exploration of this hypothesis requires the teasing apart of epigenetic from genetic effects, a challenging task in natural populations of sexually reproducing organisms in which genetic and epigenetic variation may be closely intertwined (Bossdorf & Zhang, 2011; Herrera & Bazaga, 2011). In this respect, heterophyllous plants emerge as particularly favourable study systems for the investigation of the possible epigenetic underpinnings of phenotypic plasticity. As different leaf types on the same individual are produced by the same genotype, heterophyllous plants allow epigenetic correlates of plasticity to be easily explored, at the same time as keeping DNA sequence constant, and, more generally, allow the investigation of whether epigenetic variation plays some mechanistic role in the promotion of organ-level, subindividual phenotypic plasticity (Herrera, 2009). In other words, a comparison of epigenetic features of different leaf types borne by heterophyllous plants can reveal associations between purely epigenetic variation and alternative phenotypic variants. In this article, we adopt this approach to examine whether prickly and nonprickly leaf types produced by heterophyllous European holly trees (*Ilex aquifolium* L.) differ in epigenetic features as described by their DNA methylation profiles. Heterophylly of *I. aquifolium*, which involves the facultative production of prickly leaves, is a plastic response to mammalian herbivory (Obeso, 1997). We were thus also interested in determining whether leaf phenotype and herbivory covaried in our study population. By documenting here, for the first time, a correlation between herbivory-induced heterophylly and leaf DNA methylation profile, our results provide additional support for the emerging three-way relationship between herbivory, phenotypic plasticity and epigenetic changes in plants (Verhoeven *et al*., 2010; Herrera & Bazaga, 2011; Scoville *et al*., 2011), and also contribute to the crystallization of the consensus that epigenetic variation can complement genetic variation as a source of phenotypic variation in natural plant populations (Johannes *et al*., 2009; Paun *et al*., 2010; Roux *et al*., 2011; Scoville *et al*., 2011).

MATERIAL AND METHODS

Study plant

Ilex aquifolium (Aquifoliaceae) is a small evergreen tree distributed over north-western, central and southern Europe and North Africa, where it is found associated with a broad variety of soils and plant community types (Peterken & Lloyd, 1967). Leaves can be either prickly, with a variable number of tough spines along the margin, or nonprickly with entire margins (Dormer & Hucker, 1957). As in other spinescent plants (e.g. Milewski, Young & Madden, 1991; Gómez & Zamora, 2002), the production of prickly leaves in *Ilex* L. is a plastic defensive response induced by mammalian browsing, which may subsequently reduce herbivory (Supnick, 1983; Potter & Kimmerer, 1988; Obeso, 1997). Although *I. aquifolium* trees sometimes bear only one leaf type (either prickly or nonprickly), individuals are typically heterophyllous and bear both prickly and nonprickly leaves on the same or different branches, the proportion of the two types depending on plant age, size and recent browsing history (Dormer & Hucker, 1957; Peterken & Lloyd, 1967; Obeso, 1997).

Study area and field methods

This study was conducted at a large *I. aquifolium* population located in Barranco Valdeazorillos, Sierra de Cazorla (Jaén province, south-eastern Spain). Plants grow there in the understorey of a mature *Pinus nigra* Arnold forest on a steep, north-facing slope. At the study population, most *I. aquifolium* plants were trees with one or a few trunks and well-defined crowns, 4–10 m deep, with bottom edges at 1.5–4.0 m above the ground. Forty trees occurring

along a 450-m transect running at roughly similar elevation (1300–1350m a.s.l.) across the population were selected for study. In each tree, 15 branchlets at different heights and compass directions in the lower third of the crown were examined to estimate the proportion of branchlets bearing prickly leaves. Many trees exhibited signs of browsing damage by ungulate mammals, presumably red deer (*Cervus elaphus*) and wild goats (*Capra pyrenaica*), which are abundant in the area. Browsing damage was concentrated on the accessible, lower crown layers. For each study tree, we measured the height above the ground of the bottom edge of the crown and determined whether the bottom portion of the crown showed signs of recent browsing damage (e.g. broken twigs, nibbled leaves). Nearly all trees studied were heterophyllous, although the proportion of branchlets in individual crowns bearing different leaf types varied widely (see Results). The proportions of examined branchlets bearing only prickly leaves, only entire leaves and a mixture of both types were 19.8%, 48.0% and 32.3%, respectively ($N = 600$ branchlets, all trees combined).

Five trees widely spaced along the transect, all of which were characterized by > 50% of branchlets in the lower third of the crown bearing a mixture of prickly and nonprickly leaves, were chosen for epigenetic analysis of leaf types. For each tree, a pair of undamaged, mature prickly and nonprickly leaves occupying adjacent nodal positions on a north-facing heterophyllous branchlet was collected, placed in a paper envelope and dried at ambient temperature in a sealed container with abundant silica gel until processing in the laboratory. To avoid confounding the effects of nodal position and prickliness on DNA methylation profiles, in three of the paired leaf samples, the prickly leaf occupied the basal position, and the reverse was true in the other two pairs. Representative pairs of prickly and nonprickly leaves occupying adjacent nodal positions in the same heterophyllous branchlet are shown in Figure 1 for four of the trees sampled for epigenetic analyses.

Laboratory methods

Leaf material was homogenized to a fine powder using a Retsch MM 200 mill and total genomic DNA was extracted from approximately 35mg of ground leaf material using a DNeasy Plant Mini Kit (Qiagen) and following the manufacturer's protocol. The DNA concentration of extracts was estimated by running electrophoreses of 5- µL aliquots on 0.8% agarose gels.

DNA methylation correlates of within-branchlet leaf dimorphism were investigated by fingerprinting prickly and nonprickly leaves sampled using a simplified version of the methylation-sensitive amplified polymorphism (MSAP) technique. This method is a modification of the amplified fragment length polymorphism (AFLP) technique, which allows the identification of methylation-susceptible anonymous 5′-CCGG sequences and assesses their methylation status by comparing band patterns obtained with paired primer combinations containing either of the isoschizomers *Hpa*II or *Msp*I (see, for example, Reyna-López, Simpson & Ruiz-Herrera, 1997; Cervera, Ruiz-García & Martínez-Zapater, 2002; Herrera & Bazaga, 2010, 2011). As we were interested in detecting DNA methylation differences between prickly and nonprickly leaves produced sequentially on the same branchlet by a given genotype (i.e. within-genotype methylation polymorphisms), rather than methylation differences between genotypes, our simplified MSAP method used only primer combinations with the methylation-sensitive *Hpa*II. *Hpa*II cleaves CCGG sequences, but is inactive when either or both cytosines are fully methylated, and cleaving may be impaired or blocked when one or both of the cytosines are hemi-methylated (McClelland, Nelson & Raschke, 1994; Roberts *et al*., 2007). In the absence of genetic (sequence) variation among DNA samples (e.g. between different leaf morphs on the same branchlet), therefore, any polymorphism of MSAP markers will reflect heterogeneity in the methylation status of the associated CCGG site (for applications of this simplified MSAP method, see Verhoeven *et al*., 2010; Herrera *et al*., 2012).

After a preliminary screening of 48 different *Hpa*II/*Mse*I primer combinations, four combinations each with two (*Hpa*II) or four (*Mse*I) selective nucleotides were finally chosen on the basis of repeatability and ease of scoring for fingerprinting leaf samples: *Hpa*II + TT/*Mse*I + CACT, *Hpa*II + TC/*Mse*I + CGCT, *Hpa*II + TA/*Mse*I + CACT, *Hpa*II + TG/*Mse*I + CACA. Analyses were performed essentially as described originally by Vos *et al*. (1995), with modifications involving the use of fluorescent dye-labelled selective primers following Applied Biosystems (2005). Fragment separation and detection were performed using an ABI PRISM 3130xl DNA sequencer, and the presence/absence of each marker in each sample was scored manually by the visualization of electropherograms with GeneMapper 3.7 software. Only fragments ≥ 150 base pairs in size were considered to reduce the potential impact of size homoplasy (Vekemans *et al*., 2002). Each leaf sample was fingerprinted twice in two fully independent MSAP runs, which used, as starting material, separate aliquots from the original DNA extracts.

Data analysis

In addition to common sources of genotyping errors associated with conventional AFLP fingerprinting

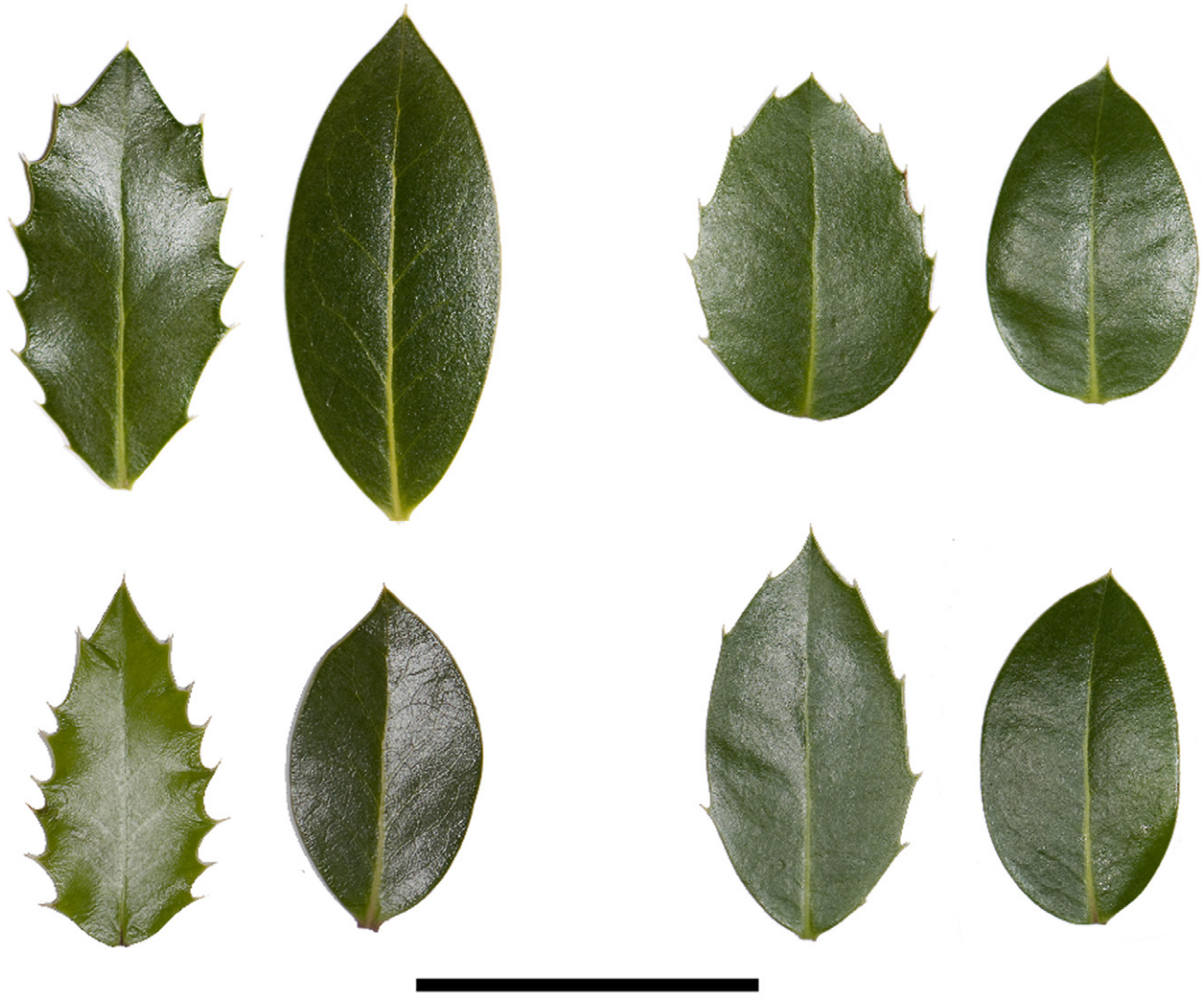

Figure 1. Pairs of prickly and nonprickly leaves borne on contiguous nodal positions of the same branchlet for four of the heterophyllous *Ilex aquifolium* trees sampled for comparative DNA methylation analyses. Scale bar = 5 cm.

(Bonin *et al.,* 2004), MSAP markers are susceptible to a stochastic component arising from within-sample heterogeneity in the methylation status of individual cytosines (see, for example, Janousek *et al*., 2002; Slotkin *et al*., 2009). This may explain why *Hpa*II-based MSAP markers are often considerably noisier than conventional, methylation-insensitive AFLP markers for the same DNA material (C.M. Herrera & P. Bazaga, unpubl. data), as denoted by a high mean per-locus mismatch rate on within-plate repeated runs (0.207 in the present study). The reduction of noise by selecting only those markers with the lowest mismatch rates may lead to informative markers being discarded, thus reducing the statistical power (Whitlock *et al.,* 2008). Instead, we adopted a statistical approach that explicitly allowed for the occurrence of a stochastic component in the data. We tested the association between leaf type and mean genome-wide methylation level by including all data from the two independent MSAP repetitions regardless of per-marker mismatch rates, and then modelling marker presence as a binomial process using a generalized linear mixed model framework (Jiang, 2007). This method (see also Verhoeven *et al*., 2010; Herrera *et al.,* 2012) is well suited to test for the significance of effects of interest on mean per-marker methylation probability because of the property of linear models of taking into account the uncertainty in the dependent variable arising from unobservable random errors (Jiang, 2007). A generalized linear mixed model was fitted to the data matrix, which consisted of presence/absence data of individual MSAP markers in the 20

samples analysed (five trees × two leaf types × two independent analytical repetitions). Marker presence (1/0) was the dependent variable, and leaf type, tree and their interaction were included as fixed effects. As scores for a given marker are expected to be correlated across samples and across repeated MSAP runs on DNA aliquots from the same leaf extract, the model included markers and replicates as random effects. The treatment of markers as random effects also ensured adequate statistical control on between-marker variation in repeatability. The assumption of marker independence implicit in our analytical layout was deemed reasonable in view of the frequent finding of AFLP markers being fairly uniformly, independently distributed across plant genomes (e.g. Castiglioni *et al*., 1999; Chagné *et al*., 2002). Computations were performed using the SAS procedure GLIMMIX, with binomial distribution for errors, logit as link function, residual pseudo-likelihood estimation and the default containment method for the computation of denominator degrees of freedom (SAS Institute, 2006). Model-adjusted least-squares means and standard errors of the response variable for the two leaf types were obtained with the LSMEANS statement and the ILINK option.

A model-free nonparametric method was used to determine whether differences between prickly and nonprickly leaves in DNA methylation occurred randomly across the genome or affected predominantly certain specific markers. We applied a recursive partitioning method based on random forests (Breiman, 2001; Hastie, Tibshirani & Friedman, 2009; for applications of random forests in genomics and ecology, see, for example, Bureau *et al*., 2005; Cutler *et al*., 2007) to identify all individual MSAP markers that were relevant to the binary classification of leaves into prickly and nonprickly classes. This ensemble learning method is particularly well suited to two-class datasets, such as the present set, where the number of attributes (markers) is considerably greater than the number of observations (DNA samples) (Strobl, Malley & Tutz, 2009). The random forests algorithm is based on the generation of a set, or 'ensemble', of classification (or regression) trees obtained on random subsets of the original data, and the identification of those attributes that are most important for classification by ranking them according to the loss of accuracy of classification caused by the random permutation of attribute values between samples (Breiman, 2001; Bureau *et al*., 2005). We performed computations with the Boruta package (Kursa & Rudnicki, 2010) for the R environment (R Development Core Team, 2010), which provides a wrapper built around the random forest classification algorithm implemented in the package randomForest (Liaw & Wiener, 2002). Boruta performs 'all-relevant feature selection', which means the identification of all attributes that are, in some circumstances, relevant for the classification (Kursa & Rudnicki, 2010). The importance of each attribute in the classification is measured by its Z score, and its significance is determined by comparison with corresponding Z values obtained from ensembles of randomized samples ('shadow' attributes), which reduces the misleading impact of random fluctuations and correlations in the data (Kursa & Rudnicki, 2010). Simulated trees are independently constructed using bootstrap samples of the dataset. The robustness of our results to random fluctuations was checked by running 20 repetitions of the analysis with different initial seeds for random tree generation. Importance ranking of markers and the identity of the subset of markers that contributed significantly to classification were closely consistent across repetitions. Only the results of one arbitrarily chosen repetition are shown here.

RESULTS

Heterophylly and herbivory

Thirty-nine of the 40 trees surveyed (97.5%) were heterophyllous, the remaining tree bearing exclusively spiny leaves in all the branchlets examined. Trees differed widely in the proportion of branchlets bearing prickly leaves (range, 6.7–100%; mean±SE, 52.0±5.2 %), and such variation was significantly related to individual differences in browsing and height above the ground of the bottom of the crown. The proportion of branchlets bearing prickly leaves was much higher among browsed (76.2±4.1%, $N = 27$ trees) than unbrowsed (19.2±2.5%, $N = 13$ trees; $\chi^2 = 28.3$, d.f. $= 1$, $P < 0.0001$, Wilcoxon rank-sum test) (Fig. 2) trees, and was inversely correlated with the height above the ground of the bottom of the crown ($r_s = -0.698$, $N = 40$, $P < 0.0001$). Unsurprisingly, ungulate browsing was most frequent among trees with crowns closer to the ground (Fig. 3). The inverse relationship that existed between leaf prickliness frequency and crown separation from the ground could thus be a spurious, indirect consequence of the fact that lower crowns experience more browsing damage, rather than a direct reflection of an architectural correlate. This possibility is strongly supported by the fact that the correlation between the proportion of branchlets with prickly leaves and the height of the bottom of the crown vanished when it was partialled on the occurrence of browsing ($r_s = -0.043$, $N = 40$, $P = 0.80$). Ungulate damage, rather than the bottom height of the crown, was therefore the best single predictor of variation among trees in the proportion of branchlets with prickly leaves.

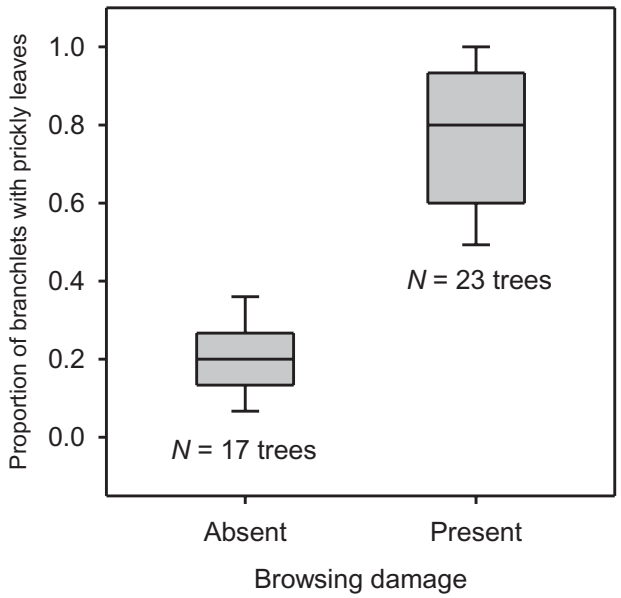

Figure2. Variation in the proportion of branchlets bearing prickly leaves in *Ilex aquifolium* trees with and without signs of browsing damage by large mammals. In each boxplot, the lower and upper boundaries of the box indicate the 25th and 75th percentiles, the line within the box marks the median, and whiskers indicate the 10th and 90th percentiles of distributions.

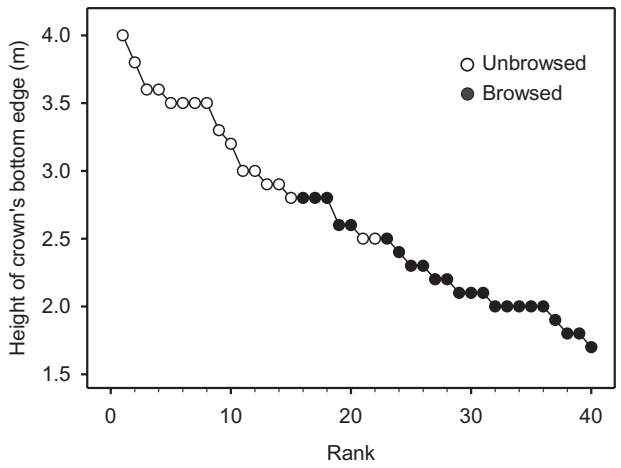

Figure3. Relationship between height above the ground of the crown's bottom edge and likelihood of damage by browsing mammals in the sample of $N = 40$ *Ilex aquifolium* trees studied. Each symbol corresponds to a single tree. Trees are ranked in decreasing order of the bottom edge height of the crown, and coded according to whether signs of recent browsing damage were present or not at the lower third of the crown. All crowns with bottoms under 2.5 m were browsed, whereas all those with bottoms above 2.8 m escaped mammalian browsers.

Table1. Summary of results of the generalized linear mixed model fitted to methylation-sensitive amplified polymorphism (MSAP) fingerprint data for DNA samples of heterophyllous *Ilex aquifolium* trees. In this model, within-genotype methylation polymorphism (*Hpa*II/*Mse*I marker presence) was the dependent variable, leaf type (prickly, nonprickly) and individual trees were treated as fixed effects, and MSAP marker and analytical run were treated as random effects

Effects Fixed	*F*	d.f.	*P* value
Leaf type (L)	7.12	1, 3353	0.0077
Tree (T)	0.24	4, 3353	0.91
L × T	1.37	4, 3353	0.24
Random	**Variance**	**Standard error**	
MSAP marker	2.2154	0.2988	
Run	0.1894	0.2725	

Heterophylly and DNA methylation

The four *Hpa*II/*Mse*I primer combinations assayed produced a total of 221 MSAP markers that could be reliably scored (see Supporting Information Data File S1). Only the 177 markers that were present in 15–90% of the 20 DNA samples analysed were retained for the comparison of cytosine methylation between prickly and nonprickly leaves.

The generalized linear mixed model testing for the effect of leaf type on MSAP score fitted the data closely, as shown by the ratio of the generalized χ^2 statistic to degrees of freedom (d.f.) close to unity (0.85). MSAP marker scores were significantly related to leaf type (Table1). After statistically accounting for the influence of random effects (marker and analytical run), the model-adjusted mean probability of MSAP marker presence was significantly higher for prickly (mean±SE = 0.681±0.072) than for nonprickly (0.632±0.077) leaves. As the presence of a marker denotes that it is in a demethylated state, these results reveal that the genome-wide, mean per-marker probability of methylation decreased by 0.049 from nonprickly to prickly leaves or, in other words, that, on average, the genome of a prickly leaf was significantly demethylated in relation to the nearest nonprickly leaf on the same branchlet. Neither the plant nor the plant × leaf type effects on MSAP marker scores were statistically significant (Table1), thus denoting homogeneity among trees in overall methylation levels and in the difference between prickly and nonprickly leaves in methylation level.

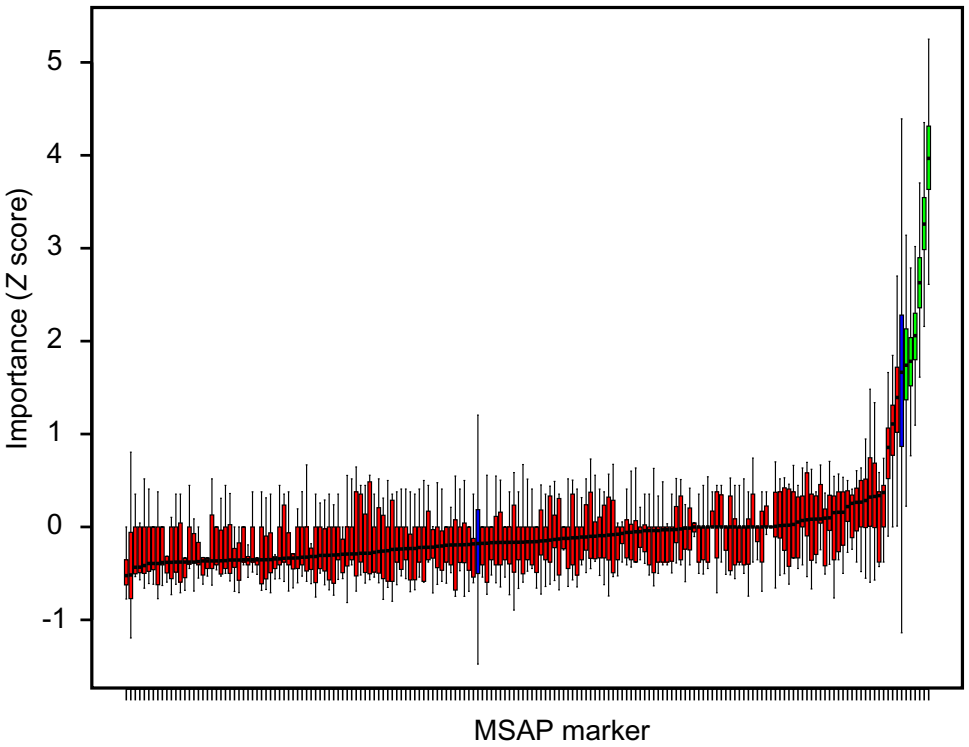

Figure4. Results of the random forests classification of DNA samples from prickly and nonprickly *Ilex aquifolium* leaves on the basis of their scores for the 177 methylation-sensitive amplified polymorphism (MSAP) markers analysed. Green and red boxplots represent *Z* scores of confirmed (i.e. contributing significantly to sample classification) and rejected (i.e. nonsignificant) markers, respectively. Blue boxplots correspond to average and maximum *Z* scores of randomly simulated ('shadow') markers. The six markers contributing significantly to the discrimination between prickly and nonprickly leaves are as follows, identified by primer combination and fragment size (base pairs): *Hpa*TT_*Msp*CACT_162, *Hpa*TT_*Msp*CACT_214, *Hpa*TC_*Msp*CGCT_157, *Hpa*TC_*Msp*CGCT_225, *Hpa*TC_*Msp*CGCT_227 and *Hpa*TA_*Msp*CACT_175.

The random forests analysis identified six MSAP markers, or 3.4% of the total, whose importance for the classification of leaf types stood apart from the rest and were deemed to contribute significantly to the classification of leaf DNA samples into nonprickly and prickly classes (Fig. 4). This result demonstrates that DNA methylation differences between leaf types, rather than being randomly spread across the genome, affected predominantly certain specific markers.

DISCUSSION

A considerable number of studies support the interpretation that increased plant spinescence, in the form of denser, longer or tougher prickles and spines in stems or leaves, represents a plastic response of plants to herbivory by large browsers, typically mammals (e.g. Bazely, Myers & da Silva, 1991; Milewski *et al.*, 1991; Obeso, 1997; Gómez & Zamora, 2002; Young, Stanton & Christian, 2003). In the case of heterophyllous plants, where individuals produce mixtures of spiny and nonspiny leaves, a handful of observational, experimental and phylogenetic investigations support both the role of vertebrate browsing as an inducer of increased spinescence and the adaptive value to plants of this plastic response to browsing damage (Supnick, 1983; Givnish *et al.*, 1994; Obeso, 1997; Eskildsen, Olesen & Jones, 2004). The results of the present investigation, although admittedly of a correlative nature, also support the role of browsing as an inducer of the plastic production of prickly leaves in heterophyllous *I. aquifolium*, as shown experimentally by Obeso (1997) for a northern Spanish population of the same species. In our study population, tree crowns with bottoms closer to the ground exhibited signs of browsing damage most frequently, and there was a significant association between browsing and the proportion of branchlets bearing prickly leaves, which was independent of the bottom height of the crown (i.e. individually variable prickliness was not a mere architectural effect). The distinct height threshold at

2.5m, under which crowns were invariably browsed (Fig.3), closely matched the vertical reach of 2.25m for adult red deer (*Cervus elaphus*), the largest browser occurring in the area (R. C. Soriguer, Estación Biológica de Doñana, CSIC, Sevilla, pers. comm.).

The two leaf types differed in the extent of genome-wide DNA cytosine methylation, as shown by the significant decline in mean per-marker probability of methylation from nonprickly to prickly leaves on contiguous positions of the same branchlet. Importantly, differences between leaf types in methylation level remained consistent across the individual trees sampled. As leaves in the prickly–nonprickly pair occupied different relative nodal positions (distal–basal) in the different trees sampled, between-tree consistency was not compatible with the possibility that observed differences between leaf types in DNA methylation reflected nodal position rather than leaf class. The results of random forests analysis showed that discordances between prickly and nonprickly leaf types in the methylation status of anonymous CCGG sites were not random, but predictably associated with certain markers. In addition to highlighting the potential of random forest classifiers to detect signals in genome-wide association studies with a small number of observations relative to the number of markers (Lunetta *et al*., 2004; Bureau *et al*., 2005), these results show that DNA methylation differences between *I. aquifolium* leaf types took place at particular zones of the genome, rather than being randomly or homogeneously distributed. The low statistical power (i.e. increased likelihood of committing a Type II error) expected from the modest sample sizes on which our epigenetic analyses were based contribute to strengthen, rather than weaken, these conclusions.

The demonstration of a causative connection between epigenetic alterations and developmental switches in leaf type in *I. aquifolium* will require experimentation involving controlled manipulation of herbivory and DNA methylation, and then testing for effects on leaf type (Bossdorf *et al*., 2010; Herrera *et al*., 2012). Two lines of circumstantial evidence, however, support the hypothesis that changes in DNA methylation play some causative, mechanistic role in the plasticity for leaf phenotype exhibited by heterophyllous *I. aquifolium* trees. First, DNA methylation in plants controls gene expression levels and is also involved in gene regulation during development (Zilberman *et al*., 2007; Gibney & Nolan, 2010; Zhang *et al*., 2011). Linkage of the MSAP markers that discriminate between prickly and nonprickly leaves to genes involved in the synthesis of hormones that regulate leaf development would provide a sufficient mechanism leading to correlations between leaf type and DNA methylation. Second, our results agree with cytological evidence presented by Bitonti *et al*. (1996, 2002) for two species of heterophyllous plants: the aquatic herb *Trapa natans* L. and the tree *Prunus persica* (L.) Batsch. In these plants, cell nuclei of meristems producing different leaf types differ in the extent of DNA cytosine methylation as evaluated by 5-methylcytidine immunocytolabelling. In *T. natans*, for example, where individuals produce contrasting floating and submerged leaves, DNA methylation was higher in floating bud meristems than in submerged ones. Our results, obtained by a different method, likewise denote leaf type-specific methylation levels.

Genome-wide changes in DNA methylation in response to changes in the environment have been increasingly shown in recent years for plants (Chinnusamy & Zhu, 2009; Peng & Zhang, 2009). Among biotic factors, herbivory has been implicated as an important ecological driver of genomic methylation changes in plants. For example, chemical induction of herbivore defences triggers considerable methylation variation throughout the genome in dandelions (*Taraxacum officinale* F.H.Wigg.; Verhoeven *et al*., 2010), individual differences in herbivory levels are related to epigenotype in a wild population of a perennial violet (*Viola cazorlensis* Gand.; Herrera & Bazaga, 2011), and epigenetic variation accounts for individual variation in response to defence hormones in *Arabidopsis thaliana* (L.) Heynh. (Latzel *et al*., 2012). The association between herbivory-induced prickly leaves and DNA methylation profiles within *I. aquifolium* plants, documented here, reveals yet another connection between herbivory and epigenetic variation. In contrast with previous investigations, however, which mostly focused on methylation differences at the whole-plant level (i.e. between genotypes), the phenotype–epigenotype correlation documented here takes place at the within-plant level. Our results are important for the following reasons. First, they further contribute to support the notion that epigenetic variation alone can be a source of phenotypic variation in natural plant populations, as demonstrated previously for cultivated plants that lack genetic variation, but exhibit substantial phenotypic variability (Fang *et al*., 2008). Second, given that within-plant variation is often the main source of population-wide variance in organ-level phenotypic traits (Herrera, 2009), the relationship found here between epigenetic differences and within-plant phenotypic variation leads to the prediction that subindividual epigenetic variation may be a major source of organ-level phenotypic variance in natural plant populations. Third, in large long-lived plants with a sectorial, compartmentalized organization (Orians & Jones, 2001), the localized action of environmental factors triggering changes in DNA methylation may generate subindividual epigenetic mosaics. In the case of *I. aquifolium*, Obeso (1997) showed that

induced responses to browsing were localized, and hence patchiness in the distribution of browsing is expected to generate concurrent patchiness in leaf methylation profiles across tree crowns in sectorially organized plants. As environmentally induced epigenetic marks with phenotypic consequences are often transgenerationally heritable in plants (Jablonka & Raz, 2009; Scoville *et al*., 2011), it is conceivable that persistent epigenetic mosaics arising within large, long-lived plants may translate into epigenetically heterogeneous progeny if induced DNA methylation marks enter the germ line and are not reset during gametogenesis (Takeda & Paszkowski, 2006; Migicovsky & Kovalchuk, 2012). This would provide yet another mechanism whereby epigenetically based phenotypic divergence could contribute to micro- and macroevolutionary change (Flatscher *et al*., 2012).

Phenotypic plasticity is expected to be particularly important when a limited control of spatial position restricts the capacity of an organism to select the features of its immediate surroundings, and hence it is not surprising that plants are remarkable for possessing it to a considerable degree (Herrera, 2009). Because of their modular organization, phenotypic plasticity in plants takes place at both the whole-plant and subindividual levels, the latter providing us with a valuable scenario for studying, in a coordinated fashion, both phenotypic plasticity and the ecological and evolutionary roles of epigenetic variation. The difficulty of obtaining sufficient replicates with identical genotypes to be tested under different environmental conditions has hindered progress in the understanding of the mechanistic basis of phenotypic plasticity. In addition, establishing the degree to which epigenetic variation is autonomous from genetic variation is central to the evaluation of the relevance of the former as an additional inheritance system (Richards, 2006; Bossdorf *et al*., 2008; Jablonka & Raz, 2009). The modular reiteration that characterizes plants allows the simultaneous circumvention of these two difficulties by providing us with ample genetically identical copies of homologous organs that represent phenotypic reruns by the same genotype under different environmental conditions (Herrera, 2009). As suggested by this study, having at our disposal a set of phenotypic variants of a given organ produced by the same (modular) genotype in response to environmental changes may allow us to unravel the role played by epigenetic modifications in delineating the phenotypic space, or 'field of possibilities' (Jorgensen, 2011), that is available to individual genotypes.

ACKNOWLEDGEMENTS

We are grateful to Esmeralda López, Mónica Medrano and Curro Molina for field and laboratory assistance; Conchita Alonso and Mónica Medrano for useful comments on the manuscript; and Consejería de Medio Ambiente, Junta de Andalucía, for permission to work in the Sierra de Cazorla. Financial support for this work was rejected by Ministerio de Ciencia e Innovación (proposal CGL2011-12534-E) and then partly granted by Estación Biológica de Doñana, which is greatly appreciated.

REFERENCES

Agrawal AA. 2001. Phenotypic plasticity in the interactions and evolution of species. *Science* **294:** 321–326.

Applied Biosystems. 2005. *AFLP plant mapping protocol*. Foster City, CA: Applied Biosystems.

Aubin-Horth N, Renn SCP. 2009. Genomic reaction norms: using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Molecular Ecology* **18:** 3763–3780.

Bazely DR, Myers JH, da Silva KB. 1991. The response of numbers of bramble prickles to herbivory and depressed resource availability. *Oikos* **61:** 327–336.

Bitonti MB, Cozza R, Chiappetta A, Giannino D, Castiglione MR, Dewitte W, Mariotti D, Van Onckelen H, Innocenti AM. 2002. Distinct nuclear organization, DNA methylation pattern and cytokinin distribution mark juvenile, juvenile-like and adult vegetative apical meristems in peach(*Prunus persica* (L.)Batsch). *Journal of Experimental Botany* **53:** 1047–1054.

Bitonti MB, Cozza R, Wang G, Ruffini-Castiglione M, Mazzuca S, Castiglione S, Sala F, Innocenti AM. 1996. Nuclear and genomic changes in floating and submerged buds and leaves of heterophyllous waterchestnut (*Trapa natans*). *Physiologia Plantarum* **97:** 21–27.

Bonin A, Bellemain E, Eidesen PB, Pompanon F, Brochmann C, Taberlet P. 2004. How to track and assess genotyping errors in population genetics studies. *Molecular Ecology* **13:** 3261–3273.

Bossdorf O, Arcuri D, Richards CL, Pigliucci M. 2010. Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology* **24:** 541–553.

Bossdorf O, Richards CL, Pigliucci M. 2008. Epigenetics for ecologists. *Ecology Letters* **11:** 106–115.

Bossdorf O, Zhang Y. 2011. A truly ecological epigenetics study. *Molecular Ecology* **20:** 1572–1574.

Breiman L. 2001. Random forests. *Machine Learning* **45:** 5–32.

Bureau A, Dupuis J, Falls K, Lunetta KL, Hayward B, Keith TP, Van Eerdewegh P. 2005. Identifying SNPs predictive of phenotype using random forests. *Genetic Epidemiology* **28:** 171–182.

Castiglioni P, Ajmone-Marsan P, van Wijk R, Motto M. 1999. AFLP markers in a molecular linkage map of maize: codominant scoring and linkage group distribution. *Theoretical and Applied Genetics* **99:** 425–431.

Cervera MT, Ruiz-García L, Martínez-Zapater JM. 2002. Analysis of DNA methylation in *Arabidopsis thaliana* based on methylation-sensitive AFLP markers. *Molecular Genetics and Genomics* **268:** 543–552.

Chagné D, Lalanne C, Madur D, Kumar S, Frigério JM, Krier C, Decroocq S, Savouré A, Bou-Dagher-Kharrat M, Bertocchi E, Brach J, Plomion C. 2002. A high density genetic map of maritime pine based on AFLPs. *Annals of Forest Science* **59:** 627–636.

Chinnusamy V, Zhu JK. 2009. Epigenetic regulation of stress responses in plants. *Current Opinion in Plant Biology* **12:** 133–139.

Cook SA, Johnson MP. 1968. Adaptation to heterogeneous environments. I. Variation in heterophylly in *Ranunculus flammula* L. *Evolution* **22:** 496–516.

Cutler DR, Edwards TC, Beard KH, Cutler A, Hess KT, Gibson J, Lawler JJ. 2007. Random forests for classification in ecology. *Ecology* **88:** 2783–2792.

DeWitt TJ, Scheiner SM, eds. 2004. *Phenotypic plasticity. Functional and conceptual approaches*. Oxford: Oxford University Press.

Dormer KJ, Hucker J. 1957. Observations on the occurrence of prickles on the leaves of *Ilex aquifolium*. *Annals of Botany* **21:** 385–398.

Eskildsen LI, Olesen JM, Jones CG. 2004. Feeding response of the Aldabra giant tortoise (*Geochelone gigantea*) to island plants showing heterophylly. *Journal of Biogeography* **31:** 1785–1790.

Fang J, Song C, Zheng Y, Qiao Y, Zhang Z, Dong Q, Chao CT. 2008. Variation in cytosine methylation in clementine mandarin cultivars. *Journal of Horticultural Science and Biotechnology* **83:** 833–839.

Flatscher R, Frajman B, Schönswetter P, Paun O. 2012. Environmental heterogeneity and phenotypic divergence: can heritable epigenetic variation aid speciation? *Genetics Research International* **2012**: doi: 10.1155/2012/698421.

Friedmann F, Cadet T. 1976. Observations sur l'hétérophyllie dans les Îles Mascareignes. *Adansonia, Serie 2* **15:** 423–440.

Gibney ER, Nolan CM. 2010. Epigenetics and gene expression. *Heredity* **105:** 4–13.

Givnish TJ, Sytsma KJ, Smith JF, Hahn WJ. 1994. Thorn-like prickles and heterophylly in *Cyanea*: adaptations to extinct avian browsers on Hawaii. *Proceedings of the National Academy of Sciences USA* **91:** 2810–2814.

Gómez JM, Zamora R. 2002. Thorns as induced mechanical defense in a long-lived shrub (*Hormatophylla spinosa*, Cruciferae). *Ecology* **83:** 885–890.

Hastie T, Tibshirani R, Friedman J. 2009. *The elements of statistical learning. Data mining, inference, and prediction*, 2nd edn. New York: Springer.

Herrera CM. 2009. *Multiplicity in unity. Plant subindividual variation and interactions with animals*. Chicago, IL: University of Chicago Press.

Herrera CM, Bazaga P. 2010. Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet *Viola cazorlensis*. *New Phytologist* **187:** 867–876.

Herrera CM, Bazaga P. 2011. Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory. *Molecular Ecology* **20:** 1675–1688.

Herrera CM, Pozo MI, Bazaga P. 2012. Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. *Molecular Ecology* **21:** 2602–2626.

Jablonka E, Raz G. 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Quarterly Review of Biology* **84:** 131–176.

Janousek B, Matsunaga S, Kejnovsky E, Ziuvova J, Vyskot B. 2002. DNA methylation analysis of a male reproductive organ specific gene (MROS1) during pollen development. *Genome* **45:** 930–938.

Jiang J. 2007. *Linear and generalized linear mixed models and their applications*. New York: Springer.

Johannes F, Porcher E, Teixeira FK, Saliba-Colombani V, Simon M, Agier N, Bulski A, Albuisson J, Heredia F, Audigier P, Bouchez D, Dillmann C, Guerche P, Hospital F, Colot V. 2009. Assessing the impact of transgenerational epigenetic variation on complex traits. *PLoS Genetics* **5:** e1000530.

Jorgensen RA. 2011. Epigenetics: biology's quantum mechanics. *Frontiers in Plant Science* **2:** 10. doi: 10.3389/fpls.2011.00010.

de Kroon H, Huber H, Stueferj F, van Groenendael JM. 2005. A modular concept of phenotypic plasticity in plants. *New Phytologist* **166:** 73–82.

Kursa MB, Rudnicki WR. 2010. Feature selection with the Boruta package. *Journal of Statistical Software* **36:** 1–13. Available at http://www.jstatsoft.org/v36/i11.

Latzel V, Zhang Y, Moritz KK, Fischer M, Bossdorf O. 2012. Epigenetic variation in plant responses to defence hormones. *Annals of Botany* **110:** 1423–1428.

Liaw A, Wiener M. 2002. Classification and regression by randomForest. *R News* **2:** 18–22.

Lunetta KL, Hayward LB, Segal J, Van Eerdewegh P. 2004. Screening large-scale association study data: exploiting interactions using random forests. *BMC Genetics* **5:** 32. doi: 10.1186/1471-2156-5-32.

McClelland M, Nelson M, Raschke E. 1994. Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acids Research* **22:** 3640–3659.

Migicovsky Z, Kovalchuk I. 2012. Epigenetic modifications during angiosperm gametogenesis. *Frontiers in Plant Genetics and Genomics* **3:** 20. doi: 10.3389/fpls.2012.00020.

Milewski AV, Young TP, Madden D. 1991. Thorns as induced defenses: experimental evidence. *Oecologia* **86:** 70–75.

Minorsky PV. 2003. Heterophylly in aquatic plants. *Plant Physiology* **133:** 1671–1672.

Núñez-Farfán J, Schlichting CD. 2001. Evolution in changing environments: the 'synthetic' work of Clausen, Keck, Hiesey. *Quarterly Review of Biology* **76:** 433–457.

Obeso JR. 1997. The induction of spinescence in European holly leaves by browsing ungulates. *Plant Ecology* **129:** 149–156.

Orians CM, Jones CG. 2001. Plants as resource mosaics: a functional model for predicting patterns of within-plant resource heterogeneity to consumers based on vascular architecture and local environmental variability. *Oikos* **94:** 493–504.

Paun O, Bateman RM, Fay MF, Hedren M, Civeyrel L, Chase MW. 2010. Stable epigenetic effects impact adaptation in allopolyploid orchids (*Dactylorhiza*: Orchidaceae). *Molecular Biology and Evolution* **27:** 2465–2473.

Peng H, Zhang J. 2009. Plant genomic DNA methylation in response to stresses: potential applications and challenges in plant breeding. *Progress in Natural Science* **19:** 1037–1045.

Peterken GF, Lloyd PS. 1967. Biological flora of the British Isles. *Ilex aquifolium* L. *Journal of Ecology* **55:** 841–858.

Pigliucci M. 1996. How organisms respond to environmental changes: from phenotypes to molecules (and vice versa). *Trends in Ecology and Evolution* **11:** 168–173.

Pigliucci M. 2001. *Phenotypic plasticity. Beyond nature and nurture*. Baltimore, MD: John Hopkins University Press.

Potter DA, Kimmerer TW. 1988. Do holly leaf spines really deter herbivory? *Oecologia* **75:** 216–221.

R Development Core Team. 2010. *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Reyna-López GE, Simpson J, Ruiz-Herrera J. 1997. Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Molecular and General Genetics* **253:** 703–710.

Richards CL, Bossdorf O, Pigliucci M. 2010. What role does heritable epigenetic variation play in phenotypic evolution? *Bioscience* **60:** 232–237.

Richards EJ. 2006. Inherited epigenetic variation – revisiting soft inheritance. *Nature Reviews Genetics* **7:** 395–401.

Richards EJ. 2011. Natural epigenetic variation in plant species: a view from the field. *Current Opinion in Plant Biology* **14:** 204–209.

Roberts RJ, Vincze T, Posfai J, Macelis D. 2007. REBASE – Enzymes and genes for DNA restriction and modification. *Nucleic Acids Research* **35:** D269–D270.

Roux F, Colomé-Tatché M, Edelist C, Wardenaar R, Guerche P, Hospital F, Colot V, Jansen RC. 2011. Genome-wide epigenetic perturbation jump-starts patterns of heritable variation found in nature. *Genetics* **188:** 1015–1017.

SAS Institute. 2006. *The glimmix procedure, June 2006*. Cary, NC: SAS Institute. Available over the internet at http://support.sas.com/rnd/app/papers/glimmix.pdf.

Scheiner SM. 1993. Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics* **24:** 35–68.

Schlichting CD. 1986. The evolution of phenotypic plasticity in plants. *Annual Review of Ecology and Systematics* **17:** 667–693.

Schlichting CD, Pigliucci M. 1998. *Phenotypic evolution: a reaction norm perspective*. Sunderland, MA: Sinauer Associates.

Scoville AG, Barnett LL, Bodbyl-Roels S, Kelly JK, Hileman LC. 2011. Differential regulation of a MYB transcription factor is correlated with transgenerational epigenetic inheritance of trichome density in *Mimulus guttatus*. *New Phytologist* **191:** 251–263.

Sculthorpe CD. 1967. *The biology of aquatic vascular plants*. London: Arnold.

Slotkin RK, Vaughn M, Borges F, Tanurdzić M, Becker JD, Feijó JA, Martienssen RA. 2009. Epigenetic reprogramming and small RNA silencing of transposable elements in pollen. *Cell* **136:** 461–472.

Strobl C, Malley J, Tutz G. 2009. An introduction to recursive partitioning: rationale, application, and characteristics of classification and regression trees, bagging, and random forests. *Psychological Methods* **14:** 323–348.

Sultan SE. 1987. Evolutionary implications of phenotypic plasticity in plants. *Evolutionary Biology* **21:** 127–178.

Sultan SE, Spencer HG. 2002. Metapopulation structure favors plasticity over local adaptation. *American Naturalist* **160:** 271–283.

Supnick M. 1983. On the function of leaf spines in *Ilex opaca*. *Bulletin of the Torrey Botanical Club* **110:** 228–230.

Takeda S, Paszkowski J. 2006. DNA methylation and epigenetic inheritance during plant gametogenesis. *Chromosoma* **115:** 27–35.

Vekemans X, Beauwens T, Lemaire M, Roldán-Ruiz I. 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. *Molecular Ecology* **11:** 139–151.

Verhoeven KJF, Jansen JJ, van Dijk PJ, Biere A. 2010. Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytologist* **185:** 1108–1118.

Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23:** 4407–4414.

Wells CL, Pigliucci M. 2000. Adaptive phenotypic plasticity: the case of heterophylly in aquatic plants. *Perspectives in Plant Ecology, Evolution and Systematics* **3:** 1–18.

Whitlock R, Hipperson H, Mannarelli M, Butlin RK, Burke T. 2008. An objective, rapid and reproducible method for scoring AFLP peak-height data that minimizes genotyping error. *Molecular Ecology Resources* **8:** 725–735.

Winn AA. 1996. Adaptation to fine-grained environmental variation: an analysis of within-individual leaf variation in an annual plant. *Evolution* **50:** 1111–1118.

Winn AA. 1999. The functional significance and fitness consequences of heterophylly. *International Journal of Plant Sciences* **160:** S113–S121.

Wund MA. 2012. Assessing the impacts of phenotypic plasticity on evolution. *Integrative and Comparative Biology* **52:** 5–15.

Young TP, Stanton ML, Christian CE. 2003. Effects of natural and simulated herbivory on spine lengths of *Acacia drepanolobium* in Kenya. *Oikos* **101:** 171–179.

Zhang MS, Xu CM, von Wettstein D, Liu B. 2011. Tissue-specific differences in cytosine methylation and their association with differential gene expression in *Sorghum*. *Plant Physiology* **156:** 1955–1966.

Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S. 2007. Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. *Nature Genetics* **39:** 61–69.

Zotz G, Wilhelm K, Becker A. 2011. Heteroblasty – a review. *Botanical Review* **77:** 109–151.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

DataFileS1. Raw methylation-sensitive amplified polymorphism (MSAP) data for *Ilex aquifolium* leaf samples used in this study.