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# Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory

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## Abstract

**Individual variation in ecologically important features of organisms is a crucial element in ecology and evolution, yet disentangling its underlying causes is difficult in natural populations. We applied a genomic scan approach using amplified fragment length polymorphism (AFLP) markers to quantify the genetic basis of long-term individual differences in herbivory by mammals at a wild population of the violet *Viola cazorlensis* monitored for two decades. In addition, methylation-sensitive amplified polymorphism (MSAP) analyses were used to investigate the association between browsing damage and epigenetic characteristics of individuals, an aspect that has been not previously explored for any wild plant. Structural equation modelling was used to identify likely causal structures linking genotypes, epigenotypes and herbivory. Individuals of *V. cazorlensis* differed widely in the incidence of browsing mammals over the 20-year study period. Six AFLP markers (1.6% of total) were significantly related to herbivory, accounting altogether for 44% of population-wide variance in herbivory levels. MSAP analyses revealed considerable epigenetic variation among individuals, and differential browsing damage was significantly related to variation in multilocus epigenotypes. In addition, variation across plants in epigenetic characteristics was related to variation in several herbivory-related AFLP markers. Statistical comparison of alternative causal models suggested that individual differences in herbivory are the outcome of a complex causal structure where genotypes and epigenotypes are interconnected and have direct and indirect effects on herbivory. Insofar as methylation states of MSAP markers influential on herbivory are transgenerationally heritable, herbivore-driven evolutionary changes at the study population will involve correlated changes in genotypic and epigenotypic distributions.**

*Keywords*: epigenetics, genomic screening, genotype–epigenotype associations, herbivory, individual variation, methylation-sensitive amplified polymorphism (MSAP)

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## Introduction

Individual variation in ecologically important features of organisms (e.g. size, fecundity, resistance to antagonists) is the rule in natural populations. Such variation is evolutionarily important, as it provides the raw material for natural selection, conditions the evolutionary trajectory of populations and affects the 'opportunity for selection' on traits (Brodie *et al.* 1995). Individual variation is also important ecologically, because it can shape the demographic structure and dynamics of populations and communities (Weiner *et al.* 2001; Herrera & Jovani 2010). Nevertheless, it is often very difficult to predict the ecological and evolutionary effects of individual variation in natural populations because of the problems involved in disentangling its causal factors in the settings where the organisms naturally occur. This

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applies particularly to the assessment of the genetic basis of individual variation, which is a relatively straightforward task in artificially assembled populations made up of individuals of known pedigree, but that becomes very difficult when it is to be addressed in natural populations (Falconer & MacKay 1996; Lynch & Walsh 1998). Conventional quantitative genetics methods are impracticable with species that cannot be artificially crossed, propagated vegetatively or kept in captivity or with those that reproduce irregularly or have long periods until first reproduction. In addition, the expression of ecologically important traits often differs between environments; hence, estimates of the genetic component of individual variation may differ widely between wild and artificial populations of the same species (Roff 1997).

Increased availability of molecular markers has stimulated, in recent years, the development of methods for the study of the genetic basis of individual variation in unpedigreed wild populations under natural conditions (Garant & Kruuk 2005; Pemberton 2008). One of these methods, which adopts a genomic scan approach and focuses on the association across individuals between ecologically important traits and genotypes, allows the quantification of the genetic component of individual variation in wild populations when individual features and multilocus data are simultaneously available (Herrera & Bazaga 2009; Herrera in press). The method proceeds by first identifying those markers whose variation across individuals is significantly correlated with differences in the feature of interest and then estimates the proportion of total variance in the sample that is statistically accounted for by individual differences in these markers by fitting a linear model to the data. In this study, we will apply this method to investigate the genetic basis of individual differences in long-term browsing damage at a wild population of the southern Spanish violet *Viola cazorlensis* (Violaceae) monitored for two decades. Experimental analyses have provided considerable evidence that individual variation in resistance to herbivores often has a genetic component (Marquis 1992; Geber & Griffen 2003), but we are not aware of any nonmanipulative study evaluating the magnitude of such component for plants growing naturally in the field. *V. cazorlensis* provides a particularly suitable study system for dissecting the roles of ecological (i.e. environmental) and genetic sources of individual variation in herbivory, because plants growing on different substrate types differ in susceptibility to mammalian browsers (Herrera 1989, 1993).

Genetic and ecological differences between individuals may not be the sole correlates of individual variation in herbivory in nature. An increasing number of studies show that plastic responses of plants to herbivores and parasites can be transmitted as maternal effects to the progeny (Agrawal 2001, 2002; Holeski 2007; Poulin & Thomas 2008), that such effects may have an epigenetic basis related to changes in DNA methylation (Pavet *et al.* 2006; Verhoeven *et al.* 2010) and that methylation-based epigenetic alterations often have phenotypic consequences without changes in the underlying DNA sequence and are stably transmitted from parents to offspring in plants (Richards 2006; Jablonka & Raz 2009; Whittle *et al.* 2009; Verhoeven *et al.* 2010). Taken together, these findings suggest that individual variation in herbivory levels ordinarily observed in natural populations could be associated with epigenetic differences. Next to nothing is known, however, on the magnitude and ecological correlates of epigenetic variation of nonmodel organisms in natural environments, as unanimously emphasized by recent reviews (Rapp & Wendel 2005; Richards 2006; Bossdorf *et al.* 2008; Richards *et al.* 2010a,b). By looking for associations across individuals between DNA methylation patterns and magnitude of browsing damage, we will investigate whether individual variation in herbivory is linked to epigenetic variation.

In contrast to genetic correlates of individual variation in herbivory, where the direction of causality can be unambiguously assigned, the interpretation of epigenetic correlates of herbivory in wild populations is much less straightforward. Correlations across individuals between herbivory level and pattern of DNA methylation may arise from past differential herbivory having induced stable epigenetic changes in plants (e.g. through induction of defences; Verhoeven *et al.* 2010), from stable epigenetic differences among individuals influencing their resistance to herbivores or from some combination of both effects. The direction of causality underlying epigenetic–herbivory correlations can be established only through manipulative experiments (Richards *et al.* 2010b; Verhoeven *et al.* 2010), but this approach is difficult with very long-lived plants like *V. cazorlensis*. Instead, we will use inferential statistical methods based on structural equation modelling (Grace 2006) to identify the most likely causal structure linking epigenotypes and herbivory in our data. This method has the additional advantage that it allows evaluating causal structures more complex than those involving just herbivory and epigenotype. Specifically, determining the degree of autonomy of epigenetic variation in relation to genetic variation and whether adaptive changes simultaneously involve genotypes and epigenotypes are two key aspects that remain unexplored in natural populations (Richards 2006; Bossdorf *et al.* 2008; Herrera & Bazaga 2010; Richards *et al.* 2010b). These aspects will be explored here by looking for associations between genotypes and epigenotypes in the broader

context of a causal framework linking herbivory, genotype and epigenotype at the individual plant level.

## Materials and methods

### *Study system*

*Viola cazorlensis* is a perennial, suffruticose violet endemic to a few contiguous limestone mountain ranges in southeastern Spain, where it occurs as discrete populations associated with rocky outcrops, cliffs and 'islands' of sandy soils originating from weathered dolomitic limestone. In winter, the aerial parts of *V. cazorlensis* plants are reduced to inconspicuous buds on the surface of the woody rootstock. Vegetative growth starts in early spring, involving rapid production of stems, leaves and flower buds. In spring and early summer, plants have a loose, cushion-like appearance and generally do not exceed 10–12 cm in height. Stems and leaves start to wither by late summer, becoming brown and senescent in autumn. Mammalian ungulates (mainly *Cervus elaphus* and *Capra pyrenaica*) frequently cause severe damage to plants by browsing on vegetative parts, flowers and developing fruits. Additional details on *V. cazorlensis* can be found in the studies by Herrera (1989, 1993) and Herrera & Bazaga (2008, 2009, 2010).

### *Study site and field methods*

This study was conducted during 1988–2008 on the same population of *V. cazorlensis* studied by Herrera & Bazaga (2009) in the Sierra de Cazorla (Jaén province, southeastern Spain). *V. cazorlensis* plants grew there in pockets of sandy soil ('ground' hereafter), crevices of bare rocks at ground level ('rocks') and crevices in vertical or overhanging cliffs >2 m in height ('cliffs'). Substrate types differ in the frequency and magnitude of browsing by mammals, which tend to be highest on ground-growing plants and lowest on cliff-growing ones (Herrera 1989, 1993).

In April 1988, a random sample of 75 reproductive individuals of *V. cazorlensis* was chosen and marked with permanent tags. Marked plants were distributed over an area of approximately 1300 $m^2$, and the two most distant ones were 110 m apart. Marked plants grew on all substrates types available at the site. These individuals were subsequently surveyed during the flowering and fruiting season (April–June) of every year until 2008. On every season, the impact of herbivorous mammals on each marked plant was estimated as the cumulative proportion of the plant surface that had been browsed by the time of fruit ripening (late June–early July). A total of 22 marked plants died from natural, albeit unknown causes over the 20-year study period. Information on substrate type, long-term herbivore incidence and genetic and epigenetic characteristics of the 53 plants that remained alive in the spring of 2008 provides the basis for this study. Herbivory data consist of a 20-year-long series (1988–2007) of annual herbivory from ungulates for each of these plants. Data for 2008 were excluded from the analyses because leaf collections from marked plants that year for obtaining DNA samples rendered estimates of herbivory unreliable. Direct data on the longevity of *V. cazorlensis* plants are not available, but survival estimates for the set of adult plants monitored over this study suggest that this encompassed a substantial portion of individual lifetimes (Herrera & Bazaga 2009). Our 20-year data of individual browsing damage most likely provide an unbiased assessment of cumulative differences in herbivory over individual lifetimes.

In April 2008, fresh leaf material was collected from each study plants and dried immediately at ambient temperature. Total genomic DNA was extracted from approximately 35 mg of ground leaf material using DNeasy Plant Mini Kit (Qiagen) and the manufacturer protocol. For each plant, the same DNA sample was used as the starting material for the genetic and epigenetic analyses described later.

### *Genetic data*

Amplified fragment length polymorphism (AFLP) markers were used to characterize the 53 study plants genetically. The AFLP analysis was performed essentially as originally described by Vos *et al.* (1995), with modifications involving the use of fluorescent dye-labelled selective primers following Applied Biosystems (2005). Each plant was fingerprinted using eight *Eco*RI + 3/*Mse*I + 3 and eight *Pst*I + 2/*Mse*I + 3 primer combinations. Fragment separation and detection were made using an ABI PRISM 3100 DNA sequencer, and the presence/absence of each marker in each individual plant was scored manually by visualizing electropherograms with GeneMapper 3.7 software. Only fragments ≥150 base pairs in size were considered, as a way of reducing the potential impact of size homoplasy (Vekemans *et al.* 2002). With only minor variations derived from the inclusion of one additional plant, the AFLP data set used here is the same that formed the base of the earlier study on this population by Herrera & Bazaga (2009), where details on primer combinations, number of markers, scoring error rates, and levels of polymorphism can be found.

### *Epigenetic analyses*

Epigenetic characterization of study plants was achieved by means of methylation-sensitive amplified

polymorphism (MSAP) analyses, which identify methylation-susceptible anonymous 5′-CCGG sequences and assess their methylation status. The MSAP method is a modification of the standard AFLP technique that uses *Eco*RI as rare cutter and *Hpa*II and *Msp*I as frequent cutters, the latter being a pair of isoschizomers that recognize the same tetranucleotide 5′-CCGG but have differential sensitivity to methylation at the inner or outer cytosine (Reyna-López *et al.* 1997; Cervera *et al.* 2002). *Hpa*II is inactive if one or both cytosines are methylated at both DNA strands, but cleaves when one or both cytosines are methylated in only one strand. *Msp*I, in contrast, cleaves $C^{5m}CGG$ but not $^{5m}CCGG$ (McClelland *et al.* 1994). Differences in the products obtained with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I should thus reflect different methylation states at the cytosines of the CCGG sites recognized by *Hpa*II or *Msp*I, which renders MSAP an efficient method for detecting alterations in cytosine methylation in plants (Cervera *et al.* 2002; Keyte *et al.* 2006). MSAP assays were performed on DNA samples from the 53 marked individuals using eight different *Eco*RI + *Hpa*II/*Msp*I paired primer combinations (Table 1).

Epigenetic fingerprinting of individual plants followed the methods described by Herrera & Bazaga (2010). For every individual and particular fragment, it was first determined whether the fragment was (i) present in both *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I products; (ii) absent from both *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I products; or (iii) present only in either *Eco*RI-*Hpa*II or *Eco*RI-*Msp*I products. Condition (i) denotes a nonmethylated state, condition (iii) corresponds to a methylated state, and condition (ii) is uninformative, as it could be attributed to either fragment absence or hypermethylation (Xiong *et al.* 1999; Ashikawa 2001; Cervera *et al.* 2002). Individual fragments were then classed as 'methylation-susceptible' ones when the observed proportion of discordant *Hpa*II-*Msp*I scores suggestive of methylation (i.e. number of plants with contrasting *Hpa*II-*Msp*I scores for the fragment divided by total number of individuals assayed) exceeded the expected per-individual probability of obtaining a mismatch of *Hpa*II and *Msp*I scores because of scoring errors alone, i.e. drawing a false inference of methylation (see Table 1 for combination-specific scoring error rates and associated methylation assignment thresholds). Methylation-susceptible fragments were scored as if the methylated state were an imperfectly assessed dominant marker: 1, for the methylated state (condition iii); 0, for the nonmethylated state (condition i); and unknown (i.e. score missing) for uninformative condition ii above.

### *Data analyses*

Band-based strategies (sensu Bonin *et al.* 2007) were adopted for the statistical analyses of both conventional AFLP and MSAP results. We searched for genetic correlates of long-term herbivory levels by running separate logistic regressions for each AFLP locus with band presence as the dependent binary variable and cumulative

**Table 1** Primer combinations used, number of MSAP markers in the size range 150–500 bp, scoring error rates and frequency and polymorphism of methylation-susceptible markers, in the methylation-sensitive amplified polymorphism (MSAP) analysis of the 53 individuals of *Viola cazorlensis* studied

| Primer combination | Total MSAP markers | Scoring error rate (%)* | | Estimated probability of erroneous *Hpa*II-*Msp*I mismatch† | Methylation-susceptible markers | |
|---|---|---|---|---|---|---|
| | | *Hpa*II | *Msp*I | | *N* | % Polymorphic§ |
| *Eco*RI + AGG/*Hpa*II-*Msp*I + TA | 35 | 4.28 | 5.00 | 0.0885 | 12 | 75.0 |
| *Eco*RI + ACC/*Hpa*II-*Msp*I + TT | 38 | 4.93 | 1.32 | 0.0612 | 13 | 69.2 |
| *Eco*RI + ACA/*Hpa*II-*Msp*I + TA | 30 | 4.58 | 1.67 | 0.0609 | 13 | 100.0 |
| *Eco*RI + AGC/*Hpa*II-*Msp*I + TC | 33 | 1.14 | 4.54 | 0.0557 | 11 | 63.6 |
| *Eco*RI + AGA/*Hpa*II-*Msp*I + TG | 58 | 3.02 | 6.03 | 0.0868 | 23 | 60.9 |
| *Eco*RI + AG/*Hpa*II-*Msp*I + TGA | 30 | 3.33 | 5.42 | 0.0838 | 10 | 90.0 |
| *Eco*RI + ACT/*Hpa*II-*Msp*I + TC | 36 | 2.78 | 5.90 | 0.0835 | 15 | 73.3 |
| *Eco*RI + AC/*Hpa*II-*Msp*I + TAA | 57 | 1.32 | 4.17 | 0.0537 | 21 | 90.5 |
| All combined | 317 | 3.03 | 4.37 | 0.0714 | 118 | 77.1 |

*From data of eight individuals that were re-assayed, calculated as 100 × (number of discordant scores on two independent analyses)/(number of scored markers × number of individuals) (Herrera & Bazaga 2009).

†Estimated average probability of obtaining discordant *Eco*RI–*Hpa*II and *Eco*RI–*Msp*I scores for an individual x locus combination because of scoring error alone, estimated from the scoring error rates for *Eco*RI–*Hpa*II (= $e_{Hpa}$) and *Eco*RI–*Msp*II (= $e_{Msp}$) as $e_{Hpa} + e_{Msp} - 2\, e_{Hpa}\, e_{Msp}$.

§A methylation-susceptible marker was considered polymorphic when both methylated and nonmethylated states occurred in the sample of individuals studied.

20-year herbivory level as the independent one (see Bonin *et al.* 2007 for the application of logistic regression as a band-based strategy for detecting AFLP marker–phenotype associations). Analyses were conducted with SAS procedure LOGISTIC (SAS Institute 2004), and the *P*-values obtained from likelihood ratio tests were used to identify statistically significant locus–fecundity associations. Analyses using original and log-transformed herbivory data led to similar conclusions, and we will report throughout results obtained with untransformed data. Given the large number of logistic regressions involved in the screening for AFLP score–herbivory associations, the possibility of obtaining an unknown number of false significant regressions (i.e. committing type I errors) should be accounted for. We applied Storey & Tibshirani's (2003) *q*-value method for the estimation of false discovery rates to the set of *P*-values for individual loci obtained from likelihood ratio tests of logistic regressions. Using the QVALUE package (Storey & Tibshirani 2003), we calculated the *q*-values for all the locus–fecundity regressions, ranked them and found the largest *q*-value leading to an expectation of less than one falsely significant regression [i.e. *q*-value × (number of regressions accepted as significant) < 1]. Multilocus genetic differences among individuals in herbivory-related loci were assessed with a principal coordinates analysis (PCA) of the genetic pairwise distance matrix between individuals, computed using exclusively those loci found to be significantly related to herbivory, as implemented in Genalex 6.1 (Peakall & Smouse 2006). Individual coordinates on the reduced dimensionality space provided by the first three PCA axes ($PC_{aflp}1$-$PC_{aflp}3$ hereafter) were then used to characterize plants from the viewpoint of their herbivory-related genetic features.

An inability to discern the cause of double-band absence is a known limitation of the MSAP method (Salmon *et al.* 2008). Because of the presence of missing scores in the MSAP data set owing to this uninformative condition, separate single-locus analyses similar to those performed for genetic data might produce spurious results if the distribution of missing scores across loci followed some hidden nonrandom pattern (e.g. if the relative causal importance of hypermethylation vs. fragment absence differed among loci). For this reason, we used multilocus analyses based on the pairwise epigenetic distance matrix, on the expectation that this multilocus characterization of individuals will be considerably more robust to possible nonrandom patterns of missing scores in the MSAP data set. It must be noted, however, that separate single-locus analyses using logistic regressions did reveal individual MSAP markers significantly related to herbivory (results not shown).

Multilocus epigenetic differences among individuals were assessed with a PCA on the epigenetic pairwise distance matrix between individuals. Individual coordinates on the reduced dimensionality space provided by the first three PCA axes ($PC_{msap}1$-$PC_{msap}3$ hereafter) were then used as a simplified characterization of the multilocus epigenotypes of individual plants. Epigenetic correlates of individual differences in herbivory levels were explored by fitting a linear model to individual data, with herbivory level as the dependent variable and $PC_{msap}1$-$PC_{msap}3$ scores as independent ones. As herbivory varied among substrates, substrate type was included in the model as an additional, categorical independent variable to statistically account for this effect.

The association between herbivory-related genetic features and multilocus epigenetic characteristics of individual plants was explored by means of distance-based redundancy analysis (dbRDA; Anderson 2003) of the epigenetic pairwise distance matrix between individuals, using individual scores on herbivory-related AFLP loci as predictor variables. Conditional (sequential) tests of the associations between individual epigenetic differences and herbivory-related AFLP loci were performed with the program DISTLM-*forward* (Anderson 2003), and statistical significance was determined using permutations.

Four alternative path models were fitted to the genetic ($PC_{aflp}1$-$PC_{aflp}3$), epigenetic ($PC_{msap}1$-$PC_{msap}3$) and herbivory data for individual plants, using SAS procedure CALIS and maximum-likelihood estimation (SAS Institute 2004). Because we were interested in the herbivory–genetic–epigenetic causal structure after controlling for ecological, substrate-related effects, the residuals after controlling for the average effect of substrate on herbivory were used as the target variable in these analyses rather than the raw herbivory data. To determine the best model(s) to describe the causal relationships among the set of intercorrelated individual features, we compared the chi-square and goodness of fit index adjusted for degrees of freedom (AGFI) of the different models (Grace 2006). The simplest possible causal model, namely one where genetic and epigenetic variables affected herbivory independently of each other, was not tested. This saturated model, equivalent to a multiple linear regression, would be uninformative because chi-square would equal zero and AGFI would be undefined because of division by d.f. = 0.

## Results

### *Individual variation in herbivory*

Individuals of *Viola cazorlensis* varied widely in both the frequency and magnitude of browsing by mammals over the 20-year study period, as denoted by differences

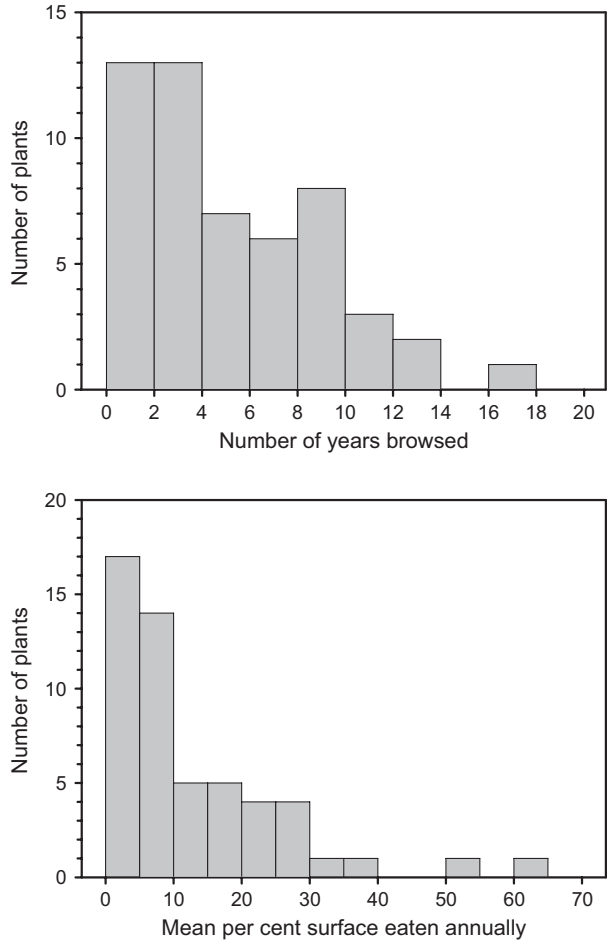


**Fig. 1** Variation in frequency and magnitude of ungulate herbivory on individual *Viola cazorlensis* plants over the 1988–2007 study period. Upper graph, frequency distribution of the total number of years (of 20) on which individual plants were browsed by mammals. Lower graph, frequency distribution of the magnitude of herbivory experienced annually by individual plants, estimated by the mean proportion of a plant's surface browsed every year.

in the number of seasons on which each plant was browsed and the mean proportion of plant surface eaten annually (Fig. 1). Some plants were never browsed in the course of the study, while one plant was browsed on as many as 16 different years. Individual differences in per-year browsing probability were statistically significant ($F_{52,1005} = 2.44$, $P < 0.0001$; tested by fitting a generalized linear model to the data, and treating browsing of a plant in a given year as a binary response binomial process). The mean proportion of plant surface browsed annually also ranged widely between individuals (range = 0–63.8%), and individual differences were statistically significant ($\chi^2 = 230.4$, d.f. = 52, $P < 0.0001$; Wilcoxon rank-sum test). Frequency of browsing and mean proportion of surface browsed annually were closely correlated across individuals ($r_s = 0.962$, $N = 53$, $P < 0.0001$). As denoted by the concave curvilinear relationship linking the two variables (Fig. S1, Supporting information), plants browsed more often over the years were also characterized by suffering a greater damage on each occasion.

### *Ecological correlates of herbivory*

On average (± SE), individual plants growing on cliffs ($N = 11$), rocks ($N = 19$) and ground ($N = 23$) were browsed on 1.6 ± 0.4 year, 5.2 ± 0.7 year and 5.8 ± 0.9 year, respectively ($\chi^2 = 9.6$, d.f. = 2, $P = 0.008$; Wilcoxon rank-sum test). This trend was paralleled by substrate-related differences in the magnitude of browsing damage. The average proportion of plant surface eaten annually for plants on cliffs, rocks and ground was 4.3 ± 1.2%, 12.9 ± 1.9%, and 17.2 ± 3.5%, respectively ($\chi^2 = 9.18$, d.f. = 2, $P = 0.01$; Wilcoxon rank-sum test). Partitioning individual variance in herbivory into its within- and among-substrate components indicated that type of substrate accounted for 22.9% and 17.0% of total variance in frequency (number of years browsed) and magnitude (mean per cent surface eaten annually) of browsing, respectively.

Given the close correlation between frequency and magnitude of browsing across individuals and substrate types, only the magnitude of browsing will be considered hereafter as a descriptor of the incidence of herbivory.

### *Genetic correlates of herbivory*

Long-term individual variation in the magnitude of browsing damage (mean per cent plant surface eaten annually) was significantly related to genetic differences. A total of six logistic regressions relating presence/absence of individual AFLP markers in a plant's genotype to its 20-year mean herbivory level were retained as statistically significant according to our criterion of keeping the expected number of false positives <1. Characteristics of the six AFLP fragments involved, and estimates and standard errors of regression parameters are shown in Table 2. These herbivory-related markers represented 1.6% of the total of 375 markers tested. A multiple linear regression relating mean plant surface eaten annually to binary scores for these six markers was highly significant, revealing that their multilocus variation across plants accounted altogether for 50.2% of population-wide variance in herbivory level ($F_{6,46} = 9.73$, $P < 0.00001$, adjusted $R^2 = 0.502$). This strong relationship remained unchanged when the dependent variable used in the regression was the residuals after controlling for the average effect of substrate on herbivory ($F_{6,46} = 7.83$, $P < 0.00001$, adjusted

**Table 2** Results of the six statistically significant logistic regressions relating individual scores for AFLP loci (1–0, presence/absence) to the long-term mean proportion of plant surface browsed annually for the $N = 53$ study plants. Regressions model the probability of marker band presence (i.e. score = 1) as a function of herbivory; hence, positive regression parameters denote a positive effect of the dominant allele and vice versa

| AFLP marker# | Primer combination (marker size, base pairs) | Regression parameter (SE) | Likelihood ratio test | |
|---|---|---|---|---|
| | | | Chi-square | *P*-value |
| 74 | PstAC-MseCAT (272) | 0.117 (0.047) | 9.96 | 0.0016 |
| 80 | PstAC-MseCAT (314) | −0.075 (0.031) | 8.01 | 0.0046 |
| 86 | PstAC-MseCAT (362) | −0.524 (0.333) | 7.79 | 0.0052 |
| 184 | PstAT-MseCCT (431) | 0.125 (0.059) | 7.49 | 0.0062 |
| 257 | EcoAGA-MseCTT (181) | 0.094 (0.040) | 8.34 | 0.0039 |
| 351 | EcoAGG-MseCAT (306) | 0.105 (0.048) | 7.48 | 0.0062 |

AFLP, amplified fragment length polymorphism.

$R^2 = 0.441$), which reveals that genetic and ecological correlates of herbivory are largely independent in the sample of individuals studied.

## *Epigenetic variation and herbivory*

Of a total of 317 markers obtained in the MSAP analysis with the eight *Eco*RI/*Hpa*II-*Msp*I primer combinations, the proportion of *Hpa*II-*Msp*I discordances was lower or equal than the corresponding combination-specific threshold for 199 loci (nonmethylated loci, 62.8% of total) and exceeded the threshold for the remaining 118 loci (methylation-susceptible loci hereafter, 37.2% of total) (Table 1). This means that about one-third of the loci obtained from the MSAP analysis were in a methylated state in a significant fraction of the individuals studied.

There was considerable epigenetic variation among individuals. Of 118 methylation-susceptible markers identified, 91 exhibited variable methylation states, or 77.1% epigenetic polymorphism (Table 1). The epigenetic pairwise distance matrix between individuals based on the scores of the 91 polymorphic epigenetic markers was subjected to a principal coordinates analysis. The first three axes ($PC_{msap}1$-$PC_{msap}3$) accounted altogether for 59.6% of total epigenetic variance among individuals. The distribution of individuals over the $PC_{msap}1$-$PC_{msap}2$ plane revealed a continuous gradation of multilocus epigenotypes in the sample (Fig. 2). Epigenetic characteristics of plants were similar across substrates ($\chi^2 \leq 2.76$, d.f. = 2, $P \geq 0.25$; Wilcoxon rank-sum tests for heterogeneity of $PC_{msap}1$, $PC_{msap}2$ and $PC_{msap}3$ scores).

Plant epigenotype was significantly related to browsing damage. The linear model testing for the relationship between substrate type and $PC_{msap}1$-$PC_{msap}3$ on one side and the magnitude of browsing on the other was statistically significant ($F_{5,47} = 3.78$, $P = 0.006$, adjusted $R^2 = 0.287$). There was a significant relationship between $PC_{msap}1$ scores and the magnitude of browsing experienced by individual plants ($F_{1,47} = 9.42$, $P = 0.004$) (Fig. 3). This herbivory–epigenotype relationship held irrespective of substrate type, as revealed by the statistical nonsignificance of the $PC_{msap}1 \times$ substrate interaction ($F_{2,47} = 0.70$, $P = 0.50$). The relationships of $PC_{msap}2$ and $PC_{msap}3$ with browsing were not statistically significant ($F_{1,47} \leq 0.19$, $P \geq 0.67$).

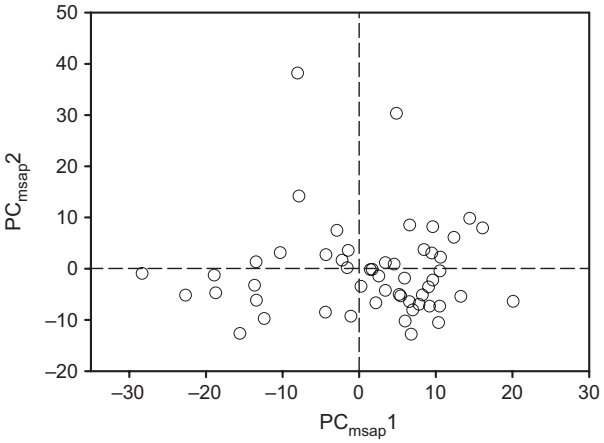


**Fig. 2** Distribution of the 53 *Viola cazorlensis* plants studied on the plane defined by the first two axes ($PC_{msap}1$ and $PC_{msap}2$) from a principal coordinates analysis of the epigenetic pairwise distance matrix between individuals. $PC_{msap}1$ and $PC_{msap}2$ account altogether for 42.7% of total epigenetic variance occurring in the sample.

## *Genotype–epigenotype associations*

Multilocus epigenotypes and herbivory-related genotypes did not covary independently across individuals in the sample studied. Table 3 summarizes the results of the distance-based redundancy analysis (dbRDA) relating the individual pairwise matrix of epigenotypic distance and the six AFLP loci significantly related to

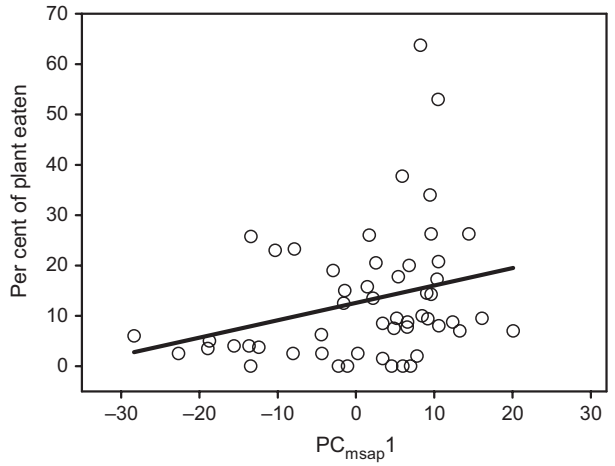


**Fig. 3** Variation in the magnitude of browsing damage experienced by individual *Viola cazorlensis* plants over the 20-year study period plotted against individual scores on the first axis from the principal coordinates analysis of the matrix of epigenetic distances between individuals ($PC_{msap}1$). Each symbol corresponds to an individual plant, and the solid line is the least-squares-fitted regression.

herbivory. Variation across plants in multilocus epigenetic characteristics was significantly related to variation in two of the six herbivory-related AFLP markers (#184 and #257) and marginally related to the variation in another (#80). Variation in these three herbivory-related AFLP markers accounted collectively for 9.2% of total epigenetic variance in the individuals sampled (Table 3).

### *Comparison of causal structures*

Four causal structures were hypothesized that could parsimoniously explain the observed associations between herbivory, genotype and epigenotype, as depicted by the simplified path models shown in Fig. 4 (see Fig. S2 for models actually fitted, Supporting information). Models differ in that epigenetic characteristics of plants are postulated to play either a causative (models 2 and 4) or consequential (models 1 and 3) role in relation to herbivory and in that epigenotypes are hypothesized as being either influenced by genotypes (models 2, 3 and 4) or independent of them (model 1). A comparison of chi-square and associated *P*-values clearly demonstrated that models 3 and 4 fitted the data much better than the other two models, which should be rejected (Table 4). Model 3 fitted the data slightly better than model 4, but the difference between their associated chi-squares and *P*-values was very small, and the fit of the two models should be considered equivalent. Comparisons of the AGFI for the different models led to similar conclusions (Table 4).

**Table 3** Distance-based redundancy analysis (dbRDA) testing for the relationship between individual epigenotypes and the six AFLP loci significantly related to long-term herbivory across the $N = 53$ individuals of *Viola cazorlensis* studied. Results correspond to conditional (sequential) tests of individual marker effects with a forward selection procedure that used the proportion of the total sum of squares explained by each marker as the criterion for selection

| AFLP marker# | Pseudo-*F* | *P*-value | Cumulative proportion of epigenetic variance explained |
|---|---|---|---|
| 184 | 1.71 | 0.018 | 0.032 |
| 257 | 1.70 | 0.017 | 0.064 |
| 80 | 1.49 | 0.059 | 0.092 |
| 86 | 1.02 | 0.43 | 0.111 |
| 351 | 0.94 | 0.56 | 0.128 |
| 74 | 0.66 | 0.89 | 0.141 |

AFLP, amplified fragment length polymorphism.

## Discussion

### *Individual variation in herbivory*

Long-term differences in browsing damage exhibited by *Viola cazorlensis* plants conform to the notion that individual variation in herbivory is an universal feature of wild-plant populations (Karban 1992; Marquis 1992). By encompassing a substantial portion of the plants' lifespan, our study differs from most previous investigations into long-lived perennials in that individual variation in browsing damage considered here most likely reflects lifetime differences. Interestingly, the extended temporal scope of this study has reinforced, rather than blurred, patterns of variation revealed by a previous 4-year study in the same population, particularly the contrast among substrates in herbivore incidence (Herrera 1993). This confirms the value of shorter-term herbivory studies in long-lived plants. The present study has also disclosed a direct relationship linking suprannual frequency and average magnitude of browsing experienced by individuals, an aspect that remains relatively unexplored in the wild populations of long-lived perennials. Plants browsed on more years also tended to suffer more extensive damage on each occasion and vice versa. This finding suggests that individual variation in resistance to browsing mainly reflects persistent individual differences in the amount or effectiveness of constitutive, rather than delayed herbivore-induced, defences against browsers. If individual variation in short-term induced defences were a major causal agent of the differences in browsing damage observed at the temporal scale of this study, then an inverse relationship between frequency and magnitude

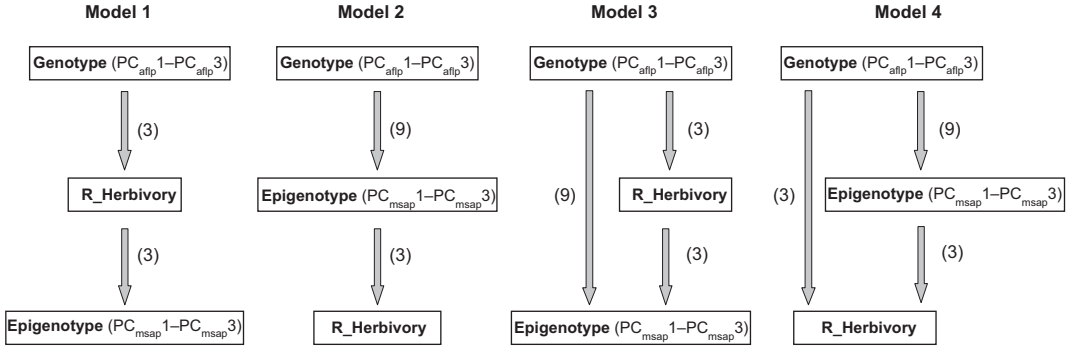


**Fig. 4** Simplified representation of the four alternative causal models linking multilocus genotypes based on herbivory-related amplified fragment length polymorphism (AFLP) markers ($PC_{aflp}1$-$PC_{aflp}3$ scores), multilocus epigenotypes ($PC_{msap}1$-$PC_{msap}3$ scores) and magnitude of browsing damage (R_Herbivory, residuals after controlling for the average effect of substrate), for the $N = 53$ *Viola cazorlensis* plants considered in this study. Each arrow denotes a causal relationship where variables at the tip are causatively influenced by variables at the base. To avoid cluttering and emphasize the conceptual structure of models, only the main causal relationships are shown here for each model, but the actual models fitted involved the three genetic and three epigenetic variables. The number of elemental causal relationships associated with each arrow and used for testing the model is shown in parentheses. See Table 4 for model fit statistics, and Fig. S2 (Supporting information) for a detailed representation of fitted models.

**Table 4** Adjusted goodness of fit index (AGFI) and chi-square tests for the four causal models shown in Fig. 4, allowing a comparison of the fit of alternative models to the data

| | Model 1 | Model 2 | Model 3 | Model 4 |
|---|---|---|---|---|
| AGFI | 0.794 | 0.602 | 0.985 | 0.978 |
| Chi-square | 19.50 | 20.52 | 0.30 | 0.44 |
| d.f. | 12 | 6 | 3 | 3 |
| *P* | 0.077 | 0.0022 | 0.96 | 0.93 |

of browsing would be expected, and the quadratic fit in Fig. S1 (Supporting information) would have been convex rather than concave.

### *Ecological and genetic sources of variation*

Variation in herbivory among *V. cazorlensis* plants reflected the combined influence of environment and genotype, as found by experimental investigations on many species (Marquis 1992; Geber & Griffen 2003). Browsing damage was significantly related to substrate type. Plants on cliffs were less damaged than those on rocks or ground, a pattern attributable to the reduced accessibility of cliff plants to browsing mammals (Herrera 1993). Similar small-scale, microhabitat-driven heterogeneity in the incidence of browsing mammals commonly occurs in plant populations, as exemplified by variations in browsing damage with distance to heterospecific neighbours or along forest understory light gradients (García & Obeso 2003; Lin & Galloway 2010). In *V. cazorlensis*, plant microhabitat differences accounted for 17% of total population-wide variance in amount of herbivory.

Long-term individual differences in herbivory had a substantial genetic component. After statistically removing the effect of substrate type, variation in the six AFLP markers significantly related to herbivory accounted for as much as 44% of population-wide variance in browsing damage experienced by individuals. The association across individuals between browsing damage and the six significant AFLP markers presumably stems from the latter being causatively related to herbivory, or linked to causative loci, through effects on phenotypic features influencing resistance to herbivores, such as the composition or effectiveness of chemical defences or deterrents. This interpretation is consistent with findings for some cultivated plants, where close linkage between AFLP markers and QTLs or genes responsible for resistance to herbivores or pathogens has been frequently found (Li *et al.* 2002; Herselman *et al.* 2004; Yang *et al.* 2004; Yuan *et al.* 2004; Stoeckli *et al.* 2009). The present results for *V. cazorlensis* seem the first example, to date, of an association between AFLP markers and herbivory in wild plants. It must also be noted that it is extremely unlikely that the relationship between herbivory level and AFLP markers was a spurious consequence of spatially patterned herbivory pressure in combination with population substructuring, because (i) a previous analysis showed that our marked plants form a single panmictic unit without detectable genetic substructuring (Herrera & Bazaga 2009); (ii) the magnitude of herbivory was not spatially patterned at the scale of this study, as evidenced by the flat variogram (Rossi *et al.* 1992) for herbivory over the 100-m distance range encompassed by marked plants (C. M. Herrera, unpublished data); and (iii) the statistical significance of the relationships between herbivory and $PC_{aflp}1$ and $PC_{aflp}3$ remained

virtually unaltered after spatial coordinates of individual plants were added to the model as covariates.

The strong genetic component underlying the resistance of *V. cazorlensis* plants to browsers exceeds most figures reported by experimental field studies of wild plants using artificially assembled sets of genotypes, where the genetic component rarely accounts for more than 10% of individual variance in herbivory (Maddox & Root 1987; Marquis 1990; Núñez-Farfán & Dirzo 1994; Roche & Fritz 1997; O'Reilly-Wapstra *et al.* 2002; Wise 2007; Johnson *et al.* 2009). This contrast possibly reflects a difference in the thoroughness of genotype sampling. While for practical reasons the majority of experimental field studies are limited to a modest number of genotypes that represent only a small fraction of all genetic variation relevant to herbivory occurring naturally, the genomic scan method used here evaluates the genetic correlates of herbivory over most of the range of relevant genetic variation found in the population. Our study plants were all different in their multilocus AFLP profiles for the six herbivory-related loci. This means that the estimate of the genetic component of herbivory was based on information from 53 different genotypes, a number not easily attained in conventional experimental field studies. Just because it allows sampling a variety of genotypes closer to the range naturally occurring in the focal population, the genomic scan approach used here (see also Herrera & Bazaga 2009; Herrera in press) is expected to provide more realistic estimates of the genetic basis of individual variation in ecologically important traits under natural field conditions than classical experimental studies, which are usually constrained to test only a modest range of genotypes.

### *Epigenetic variation and herbivory*

The potential ecological and evolutionary implications of natural epigenetic variation have been thoroughly discussed in several recent reviews, all of which have emphasized the lack of studies on natural epigenetic variation and on epigenetic effects in natural environments (Rapp & Wendel 2005; Bossdorf *et al.* 2008; Johannes *et al.* 2008; Jablonka & Raz 2009). One important result of this study thus was the discovery of a considerable pool of epigenetic variation in the local population of *V. cazorlensis* studied, as evidenced by high polymorphism of methylation-susceptible MSAP markers and broad individual differences in multilocus epigenetic characteristics. This finding confirms the results of an earlier investigation into the within- and between-population structuring of epigenetic variation in 14 populations of *V. cazorlensis* (including the present one), which showed that local populations of this species harbour, on average, 87% of regional methylation-based epigenetic variance (Herrera & Bazaga 2010). In addition, individual epigenetic variation was significantly related to long-term herbivory levels, which provides one of the few examples, to date, of ecological correlates of natural epigenetic variation in wild plants (see also Lira-Medeiros *et al.* 2010).

The association across *V. cazorlensis* plants between incidence of browsing and multilocus epigenotypes could be attributed to epigenetic differences translating into some phenotypic differences influencing plant resistance to herbivores, such as those involving the effectiveness of defences. DNA methylation in plants controls gene expression levels (Zilberman *et al.* 2007), and variation among individuals in the degree of methylation of genes can induce variation in traits such as flowering time, plant size, fecundity and resistance to pathogens or toxins (Finnegan *et al.* 1996; Sha *et al.* 2005; Giménez *et al.* 2006; Akimoto *et al.* 2007; Bossdorf *et al.* 2010). Results of the present study may be interpreted in this context. Nevertheless, the reverse interpretation, namely that differential methylation patterns are the outcome of individual differences in herbivory, could also plausibly account for the herbivory–epigenotype association found here. Exposure to environmental stresses, including herbivores and pathogens, can alter DNA methylation patterns (Madlung & Comai 2004; Pavet *et al.* 2006; Peng & Zhang 2009; Verhoeven *et al.* 2010). In the long run, sustained differences among *V. cazorlensis* individuals in mammal browsing (e.g. because of genetically based differences in quantity or quality of defences) could have induced in these long-lived plants stable epigenetic 'memories' or 'imprints' (Bruce *et al.* 2007; Berger & Chaudhury 2009; Whittle *et al.* 2009) of past herbivory levels, which would give rise to the observed correlation between herbivory and multilocus epigenotype at the population level. As noted in the introduction, assessing the merits of these two competing explanations would require manipulative experiments. Nevertheless, the comparison of alternative causal models suggests that a simple one-way directionality linking herbivory and epigenotype is unlikely in the *V. cazorlensis* population studied here, as discussed later.

### *Causal hypotheses*

Natural variation in gene methylation can be under genetic control (Zhang 2008; Jablonka & Raz 2009), and establishing the degree to which epigenetic variation is autonomous from genetic variation is important to evaluating the relevance of the former as an additional, rather than redundant, inheritance system. The evolutionary relevance of epigenetic variation will depend on epigenetic and genetic variation being largely independent of each other (Richards 2006; Bossdorf *et al.* 2008).

Little information exists, however, on the links between epigenetic and genetic variation in wild populations. In the present study, the distance-based redundancy analysis revealed a significant association between herbivory-related AFLP markers and epigenotypes, although it was relatively weak in quantitative terms (only 9% of MSAP variation explained by herbivory-related AFLP loci). In addition, the two path models providing the best fit to the data (models 3 and 4) shared a causal path whereby individual variation in genotypes directly influenced variation in both herbivory and epigenotypes, and epigenetic differences were hypothesized to be partly an effect of genetic (i.e. DNA sequence) variation. The close fit of models 3 and 4 to the data provides compelling evidence that, in the *V. cazorlensis* population studied, individual epigenetic variation was not fully autonomous from genetic variation, being at least in part a downstream, subsidiary effect of genetic variation.

Differences in fit between models 3 and 4 were too small to pick up a single best model. This result was striking, given the opposite directionalities linking epigenotypes and herbivory in the two models: herbivory influencing epigenotype in model 3 and epigenotype influencing herbivory in model 4. A parsimonious, tentative interpretation of this finding is that observed individual differences in herbivory are *simultaneously* cause and effect of variation in multilocus epigenotypes. This seemingly paradox could arise if the methylation state of some MSAP markers were modified by herbivory (e.g. labile, context-sensitive markers responsive to stress), while the methylation state of others was influential on the amount of herbivory (e.g. more stable and robust-to-stress markers associated with genes involved in constitutive defences). The multilocus approach used here could have compounded these differences, leading to a set of $PC_{msap}$ scores for each plant that are simultaneously influenced by, and influential on, herbivory. Regardless of the underlying mechanisms, however, path models have proven useful to make different causal scenarios explicit, identify models most accordant with observed data and generate testable hypotheses.

### *Evolutionary implications*

The dissection of factors underlying individual variation in herbivore incidence presented in this study prompts several evolutionary considerations. Herbivores can exert selection on plant features through discrimination among conspecifics, but the evolutionary response of plants will depend on the importance of environmental versus heritable factors in determining herbivore damage in the natural settings where the organisms occur (Karban 1992; Marquis 1992). The application in this study of a genomic scan approach to a wild-plant population has revealed an unexpectedly high genetic component underlying individual differences in mammalian herbivory in *V. cazorlensis*. As herbivory is a major factor limiting individual fecundity in *V. cazorlensis* (Herrera 1993), our results imply that adaptive responses to selection from ungulates are probably underway in the population studied. In addition, and perhaps more importantly, this study has shown that along with the customarily sought-after genetic component, individual variation in herbivory may also have a significant epigenetic component, which could also have direct and indirect evolutionary consequences. Many methylation-based epigenetic changes are transgenerationally heritable in plants and stable over several generations (Richards 2006; Akimoto *et al.* 2007; Jablonka & Raz 2009; Johannes *et al.* 2009; Whittle *et al.* 2009; Verhoeven *et al.* 2010). If the methylation states of at least some of the MSAP markers associated with the resistance of *V. cazorlensis* to mammal herbivory were heritable, then the association between epigenotype and herbivory would translate into herbivore-driven selection on epigenotypes, one key requisite for epigenetic variation to be evolutionarily relevant (Kalisz & Purugganan 2004; Jablonka & Raz 2009). Furthermore, given the significant association across individuals between multilocus epigenotypes and herbivory-related AFLP loci, the hypothesis can be advanced that herbivore-driven, evolutionary changes of genotypic and epigenotypic frequencies would not proceed independently of each other, but rather through correlated changes simultaneously involving the two inheritance layers. Such coordinated genotypic–epigenotypic changes could ultimately explain the association across populations between adaptive genetic divergence and epigenetic differentiation (Herrera & Bazaga 2010).

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## Supporting information

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

**Fig. S1** Relationship between magnitude (mean per cent plant surface browsed annually) and frequency (number of years browsed) of browsing damage experienced by individual *Viola cazorlensis* plants over the 20-year study period.

**Fig. S2** Detailed representation of the four causal models fitted to individual data for the *Viola cazorlensis* plants considered in this study, linking multilocus genotypes based on herbivory-related AFLP markers ($PC_{aflp}1$-$PC_{aflp}3$ scores), multilocus epigenotypes ($PC_{msap}1$-$PC_{msap}3$ scores) and magnitude of browsing damage (R_Herbivory, residuals after controlling for the average effect of substrate).

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