MOLECULAR ECOLOGY

Molecular Ecology (2014) **23**, 1085–1095 doi: 10.1111/mec.12679

Variation in DNA methylation transmissibility, genetic heterogeneity and fecundity-related traits in natural populations of the perennial herb *Helleborus foetidus*

CARLOS M. HERRERA, MÓNICA MEDRANO and PILAR BAZAGA

Estación Biológica de Doñana Consejo Superior de Investigaciones Científicas (CSIC), Avenida Américo Vespucio s/n, Isla de La Cartuja 41092, Sevilla, Spain

Abstract

Inferences about the role of epigenetics in plant ecology and evolution are mostly based on studies of cultivated or model plants conducted in artificial environments. Insights from natural populations, however, are essential to evaluate the possible consequences of epigenetic processes in biologically realistic scenarios with genetically and phenotypically heterogeneous populations. Here, we explore associations across individuals between DNA methylation transmissibility (proportion of methylation-sensitive loci whose methylation status persists unchanged after male gametogenesis), genetic characteristics (assessed with AFLP markers), seed size variability (within-plant seed mass variance), and realized maternal fecundity (number of recently recruited seedlings), in three populations of the perennial herb *Helleborus foetidus* along a natural ecological gradient in southeastern Spain. Plants (sporophytes) differed in the fidelity with which DNA methylation was transmitted to descendant pollen (gametophytes). This variation in methylation transmissibility was associated with genetic differences. Four AFLP loci were significantly associated with transmissibility and accounted collectively for ~40% of its sample-wide variance. Within-plant variance in seed mass was inversely related to individual transmissibility. The number of seedlings recruited by individual plants was significantly associated with transmissibility. The sign of the relationship varied between populations, which points to environment-specific, divergent phenotypic selection on epigenetic transmissibility. Results support the view that epigenetic transmissibility is itself a phenotypic trait whose evolution may be driven by natural selection, and suggest that in natural populations epigenetic and genetic variation are two intertwined, rather than independent, evolutionary factors.

Keywords: DNA methylation, epigenetic inheritance, *Helleborus foetidus*, seed size variation, seedling recruitment, within-plant variation

Received 6 August 2013; revision received 16 January 2014; accepted 22 January 2014

Introduction

Experimental studies on cultivated and model plant species conducted under artificial conditions have shown that epigenetic variation unrelated to the genetic inheritance system based on DNA sequence variants may induce phenotypic variation that is transgenerationally heritable (Jablonka & Raz 2009; Scoville *et al.* 2011; Becker & Weigel 2012). In these studies, the lack of relationship between epigenetic and genetic inheritance systems has been generally tested by comparing phenotypes of genetically identical but epigenetically distinct individuals (Johannes *et al.* 2009; Verhoeven *et al.* 2010; Becker & Weigel 2012). Without denying the critical importance of these and related experiments, research on natural populations is also needed to evaluate the significance of epigenetic processes in the scenarios where populations live and possibly evolve (Richards 2008, 2011; Richards *et al.* 2010). Restrictions

Correspondence: Carlos M. Herrera, Fax: +34 954 621125; E-mail: herrera@ebd.csic.es

imposed on genetic variation by experimental designs may convey the notion that transgenerational inheritance of epigenetically induced traits is a rather rigid, deterministic on/off switch process (Richards 2006; Becker & Weigel 2012; but see Verhoeven *et al.* 2010; Verhoeven & van Gurp 2012). In natural plant populations, however, epigenetic inheritance might be related to genetic variation, since epigenetic maintenance during gametogenesis is under genetic control (Saze *et al.* 2003; Berger & Twell 2011; Gutierrez-Marcos & Dickinson 2012) and distinct genotypes might therefore differ in important aspects of epigenetic transmission (Banks & Fedoroff 1989). Compatible with this interpretation are recent results for the perennial herb *Helleborus foetidus*, in which individuals and populations differ in the fidelity with which the methylation status of individual loci are transmitted from sporophytes to descendant gametophytes (Herrera *et al.* 2013). This highlights the importance of approaching the study of epigenetic variation with a population perspective (Richards 2008, 2011), stresses the need for examining transmissibility of epigenetic states and its implications in ecologically and genetically realistic contexts (Herrera *et al.* 2013), and supports theoretical models that consider epigenetic transmissibility and phenotypic carry-over effects across generations as variable traits exposed to selective screening by the environment (Jablonka *et al.* 1995; Lachmann & Jablonka 1996; Shea *et al.* 2011; Geoghegan & Spencer 2012, 2013).

Unravelling the relationships between genetic and epigenetic systems of inheritance has been deemed one of 'the most important problems that applied biologists, as well as evolutionary biologists, have to tackle' (Jablonka 2013, p. 104). More specifically, we contend that exploring the associations between variable epigenetic transmissibility, genetic variation and individual fitness in natural populations will be useful to evaluate whether natural selection might be acting as a driver of adaptation involving acquired, environmentally induced, epigenetically influenced phenotypic traits (Jablonka & Lamb 1995; Jablonka & Raz 2009). For example, epigenetically based local adaptation would arise in situations where contrasting environments select for different degrees of gametic transmission of epigenetically based traits (e.g. depending on environmental predictability; Lachmann & Jablonka 1996; Geoghegan & Spencer 2013), a scenario envisaged by population-epigenetic models (Jablonka *et al.* 1995; Lachmann & Jablonka 1996). These models are predicated on the implicit assumptions that (i) conspecific individuals differ in the faithfulness with which their epigenotypes are transmitted to progeny and (ii) such individual differences are associated with genetic variation, phenotypic differences and variable fitness. This framework should be ideally tested by examining variation in transmissibility of epigenetic states of loci with known phenotypic and fitness effects, but this possibility is confined to model species with detailed genetic and genomic information. Albeit suboptimally, informative tests may still be conducted on wild populations of nonmodel plants by looking for associations between transmissibility of loci susceptible to acquire variable epigenetic states, on one side, and genetic, phenotypic and fitness traits, on the other.

Adopting an observational approach, in this study we explore the associations between transgenerational transmissibility of DNA methylation, genetic characteristics, seed size phenotypes and realized fecundity across individuals and populations of *Helleborus foetidus*. Phenotypic selection on epigenetic transmissibility will occur whenever individual transmissibility differences are correlated with variations in fecundity. Seed size is a suitable focal trait to be investigated in this context, given its effects on seedling emergence and survival (Harper 1977; Silvertown 1989) and the central role played by epigenetic processes in seed growth and development (Köhler & Makarevich 2006; Xiao *et al.* 2006; North *et al.* 2010; Kesavan *et al.* 2013). Within-plant seed size variability is a major source of seed size variance in plant populations, and a phenotypic trait of potential adaptive significance because of its effects on individual fitness (Simons & Johnston 1997; Crean & Marshall 2009; Herrera 2009). Since within-plant variation in seed size cannot arise from maternal genetic heterogeneity, and the effect of heterogeneous paternity is usually negligible (Biere 1991; Castellanos *et al.* 2008), within-plant variation in seed features might reflect epigenetic heterogeneity arising from imperfect transmission of epigenetic states from the maternal to the F_1 seed generation (Banks & Fedoroff 1989; Saze *et al.* 2003; Xiao *et al.* 2006; Schmitz *et al.* 2011). This hypothesis predicts that seed size variability should be inversely related across plants to sporophyte-to-gametophyte DNA methylation transmissibility. In addition, significant associations across plants between epigenetic transmissibility, genetic characteristics, seed size variability and individual fecundity would be indicative of selection on DNA methylation transmissibility.

Materials and methods

Study plant

Helleborus foetidus L. (Ranunculaceae) is a perennial, evergreen, forest understory herb widely distributed in western Europe. Plants usually consist of several ramets, each of which produces a single terminal inflorescence after 2–7 seasons of vegetative growth.

Flowering mostly takes place during February–April. Each inflorescence produces 25–75 flowers over its 1.5- to 2.5-month flowering period. Flowers are hermaphroditic, self-compatible, extremely long-lived (up to 20 days), open gradually, and only rarely are there >5 flowers simultaneously open in each inflorescence. Bumble bees are the main pollinators. Fruit maturation and seed shedding take place in June–early-July. After falling to the ground, seeds are often dispersed by ants. In our study area (Sierra de Cazorla, Jaén province, southeastern Spain), most seeds either remain under the parent plant or are dispersed short distances by ants, and seedling recruitment beyond the close vicinity of adult plants is usually negligible (Garrido *et al.* 2007; Manzaneda & Rey 2008). Seedling mortality is the main factor limiting population growth, concentrates on the first few weeks following emergence, varies over small spatial scales due to microhabitat heterogeneity and is mostly caused by water stress (Garrido *et al.* 2005, 2007; Ramírez *et al.* 2006).

Field methods

In the Sierra de Cazorla area, *H. foetidus* is distributed over a broad range of elevations (700–1850 m a. s. l.) and forest types. To enhance the range of ecological conditions sampled, we studied three populations at low ('Tejerina', TEJ hereafter, 730 m a. s. l.), middle ('Las Navillas', NAV, 1240 m) and high ('Puerto Llano', PLL, 1800 m) elevations. Variation in winter and summer temperature, rainfall, frost periods and habitat structure along this range of elevations presumably translates into different local dynamics and selective regimes on *H. foetidus* populations (Ramírez *et al.* 2006; Garrido *et al.* 2007).

At each site, 20 widely spaced, inflorescence-bearing plants were randomly selected during February–May 2012. These 60 plants were the same studied by Herrera *et al.* (2013; see their Table S1 for plant characteristics). Elevational differences resulted in phenological differences between sampling sites. To avoid possible developmental variation in DNA methylation confounding individual or population differences in methylation patterns, samples for molecular analyses were collected at each site during the local flowering peak (February, March and May, for TEJ, NAV and PLL, respectively). Paired leaf and pollen samples were collected from each plant. Young expanding leaves were cut, placed in paper envelopes and dried immediately at ambient temperature in sealed containers with abundant silica gel. Pollen samples were collected by bagging inflorescences for ~7 days at the beginning of flowering (to prevent access to pollen-collecting bumble bees) and then holding flowers on top of microcentrifuge tubes that were vibrated manually. Samples were examined immediately after collection to remove any maternal tissue (e.g. anther walls) and dried at ambient temperature. After pollen sample collection, inflorescences were left exposed to natural pollinator visitation for the rest of their flowering period. On each marked plant, a sample of developing fruits originating from flowers exposed to natural pollination was bagged following the end of flowering, and their mature seeds collected after fruit ripening.

In late-spring 2012, we counted the number of first-year *H. foetidus* seedlings within a 0.5-m radius around each marked plant. Seeds of *H. foetidus* generally germinate during the second spring after entering the seed bank. Since all marked plants were ≥4 year old (based on counts of annual scars on stems), we assumed that first-year seedlings around a given individual most likely arose from seeds produced by that plant 1–2 years before and could be used as rough estimates of realized maternal fecundity during recent reproductive episodes. Only large seedlings bearing one or more leaves in addition to cotyledons were tallied, as these were most likely to withstand the upcoming summer drought and eventually become established in populations. Whenever possible, a sample of healthy, undamaged seedlings associated with marked plants was collected, placed individually in paper envelopes and dried at room temperature in a container with silica gel. Genetic fingerprinting with nuclear microsatellites was then used to verify their putative maternal parentage.

Laboratory methods

Total genomic DNA was extracted from dry leaf (~30 mg) and pollen (~7 mg) material using Qiagen DNeasy Plant Mini Kit and the manufacturer protocol, with some minor modifications required for processing the small volumes of pollen samples. We used methylation-sensitive amplified polymorphism (MSAP) analysis to identify methylation-susceptible anonymous 5′–CCGG sequences, and comparatively assess their methylation status in leaves and pollen of the same plant. MSAP is a modification of the standard amplified fragment length polymorphism (AFLP) technique that uses the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I in parallel runs in combination with another restriction enzyme (commonly *Eco*RI or *Mse*I; *Mse*I was used here because of better repeatability). *Hpa*II and *Msp*I recognize the same tetranucleotide 5′-CCGG but have differential sensitivity to methylation at the inner or outer cytosine. Differences in the products obtained with *Hpa*II and *Msp*I thus reflect different methylation states at the cytosines of the CCGG sites recognized by *Hpa*II or *Msp*I cleavage sites (see e.g. Herrera & Bazaga

2010; Lira-Medeiros *et al.* 2010; for applications of the MSAP technique to wild plants). MSAP assays were conducted on leaf and pollen DNA samples from the 60 *H. foetidus* plants studied, using four *Mse*I + *Hpa*II/*Msp*I primer combinations. Fragment separation and detection were made using an ABI PRISM 3130xl DNA sequencer, and the presence or absence of *Mse*I + *Hpa*II and *Mse*I + *Msp*I fragments in each sample was scored manually by visualizing electrophoregrams with GENEMAPPER 3.7 software. Genotyping error rates were computed for each fragment by running repeated analyses for 36 samples (30% of total) and estimated as the ratio of the number of discordances to the number of samples scored. Only fragments with error rates lower than the median of the error distribution for the whole set of fragments were retained for further analysis ($N = 143$). The leaf and pollen MSAP data used here to estimate DNA methylation transmissibility for each plant are the same analysed by Herrera *et al.* (2013).

In addition to the MSAP analyses of leaf and pollen material, conventional AFLP fingerprinting of leaf samples was also undertaken to characterize plants genetically. We used four *Pst*I + 2/*Mse*I + 3 primer combinations, chosen from a broader set of combinations previously assayed in a pilot study. Fragment separation and detection was made using an ABI PRISM 3130xl DNA sequencer, and the presence or absence of each AFLP fragment in each individual plant was scored manually by visualizing electrophoregrams with GENEMAPPER 3.7 software. Only fragments ≥200-base pairs in size were considered to reduce the potential impact of size homoplasy (Vekemans *et al.* 2002). Genotyping error rates were determined for each fragment by running repeated, independent analyses for six plants (10% of total) and estimated as the ratio of the number of discordant scores to the number of repetitions. A total of 103 AFLP fragments with ≤1 discordant scores in the six repetitions and present in 3–97% of samples were retained for analysis. Overall genotyping error rate for these fragments was 5.50%.

Genomic DNA was extracted from collected seedlings using Qiagen DNeasy Plant Mini Kit. All seedlings and marked plants (leaf samples) were genotyped using eight polymorphic nuclear microsatellite loci (*Hefo1*, *Hefo2*, *Hefo4*, *Hefo6*, *Hefo8*, *Hefo9*, *Hefo10* and *Hefo13*), chosen among those described by Consortium MERPD *et al.* (2013), where details on amplification conditions and PCR cycle profiles may be found. Amplified products were analysed on an ABI PRISM 3130xl DNA sequencer. Fingerprint profiles were scored by visualizing electrophoregrams with GENEMAPPER 3.7 software.

A total of 867 seeds were weighed individually to the nearest 0.1 mg after removal of the ancillary elaiosome (range = 10–15 seeds weighed per plant), and mean and variance of seed mass estimated for each plant.

Data analysis

All statistical analyses were carried out using the R environment (R Development Core Team 2012). Ordinary linear models and generalized linear models were fitted using, respectively, lm and glm functions from the stats package. The lme function from the nlme package was used to fit linear mixed-effect models. Negative binomial errors and logarithm link function were used when fitting generalized linear models to seedling count data.

Methylation-sensitive amplified polymorphism analyses of leaf and pollen samples followed the methods described by Herrera & Bazaga (2010) as implemented in the msap package (Pérez-Figueroa 2013). Following element-wise comparisons between the two presence–absence matrices of MSAP fragments obtained for each type of sample (leaf and pollen) with *Mse*I-*Hpa*II and *Mse*I-*Msp*I primer combinations, a total of 107 methylation-susceptible loci were identified. The methylation status of every fragment in each sample was determined depending on whether the fragment was present in both *Mse*I-*Hpa*II and *Mse*I-*Msp*I products (nonmethylated state), present only in either *Mse*I-*Hpa*II or *Mse*I-*Msp*I products (hemimethylated or internal cytosine methylation), or absent from both *Mse*I-*Hpa*II and *Mse*I-*Msp*I products (uninformative condition, scored as missing). Separate MSAP score matrices were obtained for leaf and pollen material.

DNA methylation transmissibility was computed for each plant as the proportion of methylation-susceptible loci whose methylation status did not change from leaf to pollen (i.e. persisted unchanged beyond meiosis and male gametogenesis; see Herrera *et al.* 2013 for further details and justification). To determine whether individual variation in DNA methylation transmissibility was associated with genetic differences, we looked for AFLP loci that were significantly associated with transmissibility in our sample. Separate linear mixed-effect models were fit for each AFLP locus using REML estimation. In these models, methylation transmissibility was the dependent variable and fragment presence–absence the single fixed-effect, two-level factor. Application of Bayesian clustering to the AFLP fingerprint data revealed that the plants sampled were genetically structured, falling into one of two genetically distinct clusters (Appendix S1, Supporting information; one cluster corresponded to TEJ plants, and the other included NAV and PLL plants). The possible confounding effect of this genetic structuring on results of the transmissibility-AFLP loci association analysis was corrected by including genetic cluster as a random effect in the models (Price *et al.* 2010). *P*-values for the effect of fragment presence–absence on transmissibility were used to

identify significant transmissibility-AFLP locus associations. Given the large number of models fit, Storey & Tibshirani's (2003) *q*-value method was applied for estimation of false discovery rates. Using the qvalue package (Storey & Tibshirani 2003), we calculated the *q*-values for all the locus-transmissibility models 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].

The assumption that first-year seedlings in the vicinity of marked plants reflected their recent maternal fecundities was verified by comparing microsatellite fingerprints of sampled seedlings with those of putative maternal parents. A simple parentage exclusion procedure was applied, parentage being rejected when the two alleles of some locus were unrepresented in the putative mother's genotype. Maximum estimated probability of maternity exclusion, obtained by applying equation 2a in Jamieson & Taylor (1997; 'probability of detecting a falsely reported parent with offspring, where the other parent is missing') to allelic frequencies of the 8 microsatellite loci was 0.92 at all sites.

Results

DNA methylation transmissibility

Plant-to-pollen (i.e. sporophyte-to-gametophyte) DNA methylation transmissibility, measured as the proportion of methylation-sensitive loci whose methylation status persisted unchanged after male gametogenesis for a given plant (termed 'transmissibility' hereafter), was 0.840 ± 0.005 (mean ± SE, $N = 60$ plants; all means are hereafter reported ±1 SE), which revealed a remarkable epigenetic constancy from plants to descendant male gametophytes. There was, however, considerable individual spread around this mean value within populations (Fig. 1). Partitioning total transmissibility variance in the 60 plant sample into between- and within-population components indicated that variation among locally coexisting individuals accounted for 73.7% of total. Population differences, although quantitatively minor, were statistically significant ($\chi^2 = 13.49$, d.f. = 2, $P = 0.0012$; Kruskal–Wallis rank sum test), largely reflecting the higher transmissibility of TEJ plants relative to those from NAV and PLL (Fig. 1).

Association between methylation transmissibility and AFLP loci

Locus-by-locus linear mixed-effect models identified four AFLP loci (3.9% of total) which, after accounting for the genetic structure in the sample, were significantly associated with methylation transmissibility

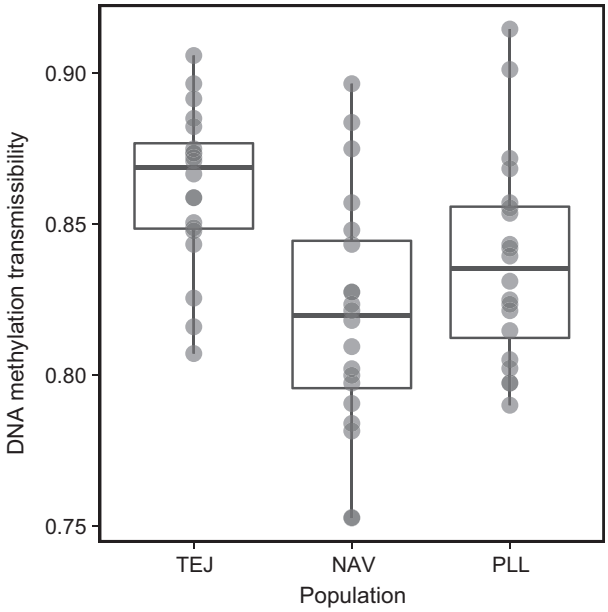

Fig. 1 Variation between *Helleborus foetidus* plants and populations (TEJ, NAV and PLL) in DNA methylation transmissibility, estimated for each plant as the proportion of methylation-sensitive loci whose methylation status persisted unchanged after male gametogenesis. Lower and upper boundaries of boxes indicate the 25th and 75th percentiles, the line within the box marks the median, and whiskers extend over the observed range. Dots represent values for individual plants.

($P \leq 0.005$, *q*-value ≤0.095; expected number of false positives = 0.095 × 4 = 0.38). AFLP marker presence was inversely related to transmissibility in three instances, and directly related in one instance (Fig. S1, Supporting information). Fitting a linear model to the transmissibility (dependent variable) and AFLP score data for the four significant loci (predictor variables) revealed that the latter accounted altogether for as much as ~40% of total between-plant variance in transmissibility ($F_{4,55} = 9.71$, $P < 0.0001$, adjusted $R^2 = 0.37$).

Seed size variability and methylation transmissibility

Individual plants of *H. foetidus* differed significantly in both the mean ($F_{58,808} = 22.02$, $P < 0.0001$) and the variance ($\chi^2 = 87.87$, d.f. = 58, $P = 0.0069$; Kruskal–Wallis robust Levene-type test on absolute deviations from the median) of their seed mass distributions. Means and variances were uncorrelated across plants ($r = -0.175$, $N = 60$, $P = 0.18$). Populations differed in individual means ($F_{2,57} = 28.91$, $P \ll 0.001$) and, marginally, individual variances ($F_{2,57} = 2.45$, $P = 0.095$) of seed mass. Plants from TEJ tended to have the heaviest seeds, those from PLL the lightest ones and those from NAV were intermediate (Fig. S2, Supporting information).

Within-plant variance in seed size tended to exhibit the opposite trend (Fig. S2, Supporting information).

A linear model was fitted to individual plant data with variance of seed mass as dependent variable, and transmissibility, population and their interaction as predictors. The model fit was statistically significant ($F_{5,54} = 3.66$, $P = 0.0064$, adjusted $R^2 = 0.184$) and revealed a significant effect of transmissibility on within-plant variance in seed mass ($F_{1,54} = 8.97$, $P = 0.0041$). Neither the population ($F_{2,54} = 1.88$, $P = 0.16$) nor the population × transmissibility interaction ($F_{2,54} = 1.74$, $P = 0.19$) effects reached statistical significance. The relationship between seed mass variance and transmissibility was an inverse, triangular one, with both the mean and the spread of within-plant variance in seed mass declining with increasing methylation transmissibility (Fig. 2).

Seedling recruitment and methylation transmissibility

First-year seedling density around *H. foetidus* plants was lowest at TEJ (mean = 2.16 ± 0.60 seedlings/m^2, $N = 20$), intermediate at NAV (4.46 ± 1.30 seedlings/m^2, $N = 20$) and highest at PLL (9.99 ± 2.51 seedlings/m^2, $N = 20$). A total of 91 seedlings from the vicinity of marked plants were fingerprinted with microsatellites (20, 33 and 38 seedlings from TEJ, NAV and PLL, respectively). After comparing fingerprints of seedlings and putative maternal parents, the inferred parentage was rejected for 20 seedlings (5, 8 and 7 from TEJ, NAV and PLL, respectively) associated with 13 different plants (5, 5 and 3 plants from TEJ, NAV and PLL, respectively). These plants were excluded from further analysis.

A generalized linear model with number of seedlings around each plant as dependent variable, and transmissibility, population and their interaction as independent ones fitted well the data (residual deviance to degrees of freedom ratio = 1.16; $F_{5,41} = 3.56$, $P = 0.0092$, Wald test). The population effect was statistically significant ($\chi^2 = 8.19$, d.f. = 2, $P = 0.017$), and the main effect of transmissibility was only marginally so ($\chi^2 = 2.81$, d.f. = 1, $P = 0.094$), which must be related to the significant population × transmissibility interaction ($\chi^2 = 6.74$, d.f. = 2, $P = 0.034$). Number of seedlings in the vicinity of a given plant was directly related to the plant's transmissibility in the TEJ site, but inversely in NAV and PLL (Fig. 3). Separate generalized linear models fits for each population revealed significant or marginally significant effects of transmissibility on number of seedlings at all sites ($P = 0.081$, 0.040 and 0.058, for TEJ, NAV and PLL, respectively), despite the small number of plants and inherently low statistical power of each analysis.

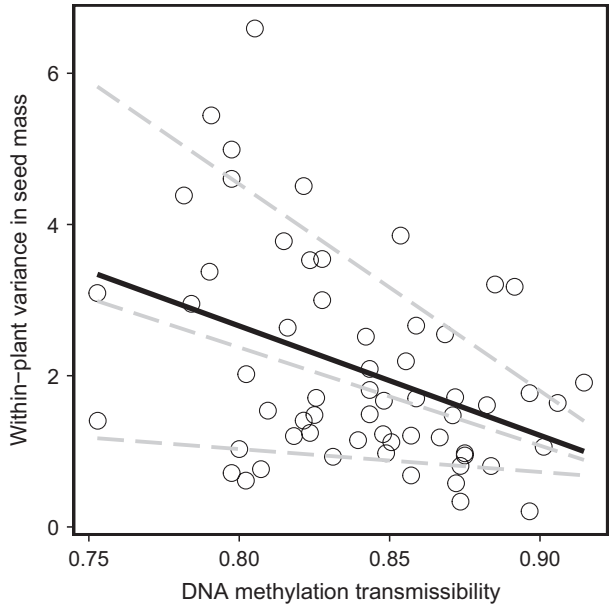

Fig. 2 Relationship between seed mass variance and DNA methylation transmissibility in the *Helleborus foetidus* plants studied. Ordinary least-squares regression (solid black) and quantile regressions (0.15, 0.50 and 0.85 quantiles; dashed grey) are shown. Quantile regressions differ significantly in slope ($F_{2,178} = 4.44$, $P = 0.013$).

Discussion

Epigenetic inheritance in plants largely relies on the maintenance of DNA cytosine methylation through meiosis and postmeiotic mitoses associated with gametogenesis (Saze *et al.* 2003; Takeda & Paszkowski 2006; Migicovsky & Kovalchuk 2012). Looking at the concordance of DNA methylation between individual plants (diploid sporophytes) and their descendant pollen (haploid gametophytes) may thus provide insights on the evolutionary significance of epigenetic phenomena in natural populations of nonmodel plants, as exemplified for *H. foetidus* by Herrera *et al.* (2013) and the present study. In the three *H. foetidus* populations studied, ~75% of MSAP loci predominantly persisted unchanged from plant to pollen ('stable loci', Herrera *et al.* 2013), and individual plants had on average ~80% of their MSAP loci unchanged (this study). These figures, which are approximately comparable to sporophyte-to-sporophyte methylation transmissibility estimates obtained for other plants under artificial conditions (Takeda & Paszkowski 2006; Verhoeven *et al.* 2010), suggest considerable *average* transgenerational inheritance of epigenetically mediated traits in natural plant populations. Beyond this confirmation of previous studies for a wild plant, our results have shown (see also Herrera *et al.* 2013) that *H. foetidus* populations harboured a broad

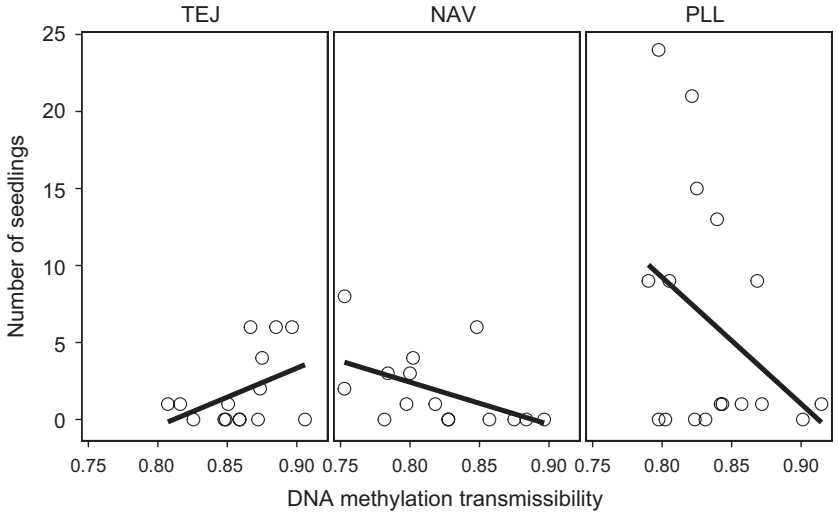

Fig. 3 Relationship between DNA methylation transmissibility of individual *Helleborus foetidus* plants and number of first-year seedlings within a 0.5-m radius, shown separately for the three study populations. Least-squares fitted regressions are shown here only to visualize between-site differences in the sign of the relationship, without any intended statistical inference meaning. See text for details of statistical analyses.

range of methylation transmissibilities (i.e. a sizeable individual *variance* around mean transmissibility). Importantly, and here lies the main novelty of the results of this study, such individual variation was significantly associated with genetic characteristics, within-plant variability in seed size, and estimated maternal reproductive success. Although admittedly of a correlative nature, these findings have a number of significant ecological and evolutionary implications.

DNA methylation transmissibility, seed size heterogeneity and genetic variation

Within-plant variation is a major source of seed size variance in plant populations, and its evolutionary persistence has been explained by 'diversified bet-hedging' models. These models predict that insofar as there is sufficient spatial or temporal uncertainty regarding the establishment success of seeds differing in size, individual plants with variable seeds will spread the risk and experience a fitness advantage over conspecifics producing homogeneous seed crops (Philippi & Seger 1989; Simons & Johnston 1997; Crean & Marshall 2009). Mechanistically, within-plant variation in seed size is the outcome of deterministic and stochastic factors operating at different levels of organization. Factors acting at the level of organs, organ systems and whole individuals include architectural effects due to plant sectoriality, resource-mediated interference between organs, and developmental instability (reviewed in Herrera 2009). Mating differences may also contribute to within-plant seed size heterogeneity in self-compatible plants with mixed mating, since selfed and outcrossed seeds often differ in size (Charlesworth & Charlesworth 1987; Manasse & Stanton 1991). The preceding 'macroscopic' factors attain their efficacy through the local action of molecular agents on the developing seeds (i.e. at tissue and cellular levels), the best known of which are phytohormones (Herrera 2009). Although infrequently acknowledged, however, it has been long known that epigenetic processes may also induce the production of phenotypically heterogeneous seed crops by individual plants (McClintock 1950; Banks & Fedoroff 1989; Das & Messing 1994).

DNA methylation plays a central role in all major processes conditioning final seed size, including cell multiplication, resource accumulation and endosperm development (Köhler & Makarevich 2006; North *et al.* 2010; Kesavan *et al.* 2013). In *Arabidopsis thaliana*, mutations in genes responsible for methylation maintenance during gametogenesis (*MET1* and *DDM1*) affect seed size; differences between parent plants or their gametes in DNA methylation level translate into significant differences in the mean size of F_1 seeds; and variable methylation in gametophytes is sufficient to generate heterogeneity in F_1 seed size (Xiao *et al.* 2006). Extensive variation in methylation between gametes produced by the same plant, both male and female, is one of the consequences of deficient expression of methylation maintenance genes (Saze *et al.* 2003). These observations suggest a parsimonious mechanism to explain the inverse relationship found here between within-plant variance in seed size and methylation transmissibility. Assuming that methylation transmissibility estimates for male gametogenesis used here are correlated with the corresponding transmissibility values for female gametogenesis (see Herrera *et al.* 2013 for review and discussion), then imperfect transmission of methylation marks from maternal parent to gametophytes will be sufficient to generate within-plant variability in seed size in *H. foetidus* as found in *Arabidopsis thaliana* (Xiao *et al.* 2006). Under this hypothesis, and for probabilistic

reasons alone, the more imperfect (i.e. noisy or unpredictable) the mother-to-gametophyte transmissions, the greater the expected within-plant variance in seed size. Our results clearly agree with this expectation. The triangular nature of the inverse seed variance-transmissibility relationship, where low transmissibilities are associated with broader scatter of data points (i.e. a looser association), lends additional support to the preceding interpretation. Scattering of points in the graph may also partly reflect that epigenetic variation observed in pollen was altered after fertilization (Johannes *et al.* 2009).

A revealing result of this study was the significant association found across *H. foetidus* plants between methylation transmissibility and four AFLP loci. Interpretation of this result is subject to the usual caveat that genetic marker-trait associations are not conclusive evidence for causality (Platt *et al.* 2010). Keeping this in mind, two mechanistic considerations lend plausibility to the interpretation that the association between genetic features and methylation transmissibility found here may stem from a causal relationship: (i) methylation maintenance during gametogenesis is under close genetic control (Saze *et al.* 2003; Xiao *et al.* 2006; Berger & Twell 2011; Gutierrez-Marcos & Dickinson 2012); and (ii) in eukaryotic genomes, a significant proportion of AFLP markers are either linked to genes of known phenotypic effects (Raman *et al.* 2002; Dussle *et al.* 2003; Herselman *et al.* 2004) or positioned within gene sequences (Caballero *et al.* 2013). Consequently, significant AFLP marker-methylation transmissibility associations found here might simply arise if the markers involved were linked to, or positioned within, genes directly controlling the fidelity of epigenetic transmission during gametogenesis (e.g. *MET1*, *DDM1*, *CMT3*; Goll & Bestor 2005).

Selection on epigenetic transmissibility and its implications

The main hypothesis considered in this study, that transmissibility of epigenetic states is an individual feature subject to selection by the environment, was addressed using a phenotypic selection approach (Lande & Arnold 1983; Endler 1986). Under this conceptual framework, selection is considered to occur whenever individuals with different characteristics (i.e. different phenotypes) differ in their survival, fecundity or mating success (Kingsolver & Pfennig 2007). Within local populations, *H. foetidus* plants differing in methylation transmissibility differed also in maternal fecundity (estimated by number of seedlings recruited), which confirms our hypothesis by demonstrating phenotypic selection on transmissibility. Interestingly, populations differed in the sign of the relationship between fecundity and methylation transmissibility, which was negative in TEJ and positive in NAV and PLL. This unanticipated result suggests that different ecological scenarios may lead to contrasting selective regimes on methylation transmissibility. The association between transmissibility and seed size variance suggests that spatially variable selection on the latter may eventually lead to variable selection on transmissibility. It may be speculated, for example, that spatio-temporal unpredictability in factors conditioning seedling emergence and establishment in *H. foetidus* (temperature, rainfall and woody cover; Garrido *et al.* 2005; Ramírez *et al.* 2006) was smaller at the lowland TEJ site than at the mid- and high-elevation localities (NAV, PLL), leading to variable selection on the breadth of within-plant seed size distributions as predicted by diversified bet-hedging models (Crean & Marshall 2009; Herrera 2009).

Imperfect transgenerational transmission of DNA methylation in plants, due to failure to faithfully maintaining genome-wide methylation patterns by *MET1* or functionally similar methyltransferases, lies at the origin of novel methylation variants and provides a key mechanism for the appearance of phenotypic diversity in the absence of genetic mutation (Schmitz *et al.* 2011). It has been postulated that the stability of epigenetic transmission is likely to be an evolved trait, that organisms have evolved mechanisms to influence epigenetically based heritable variability and that increased stochastic variation in epigenomes may enhance fitness in variable environments (Rando & Verstrepen 2007; Jablonka & Raz 2009; Feinberg & Irizarry 2010; Jorgensen 2011). Ecological factors conditioning the evolution of epigenetic inheritance systems have been also explored theoretically (Jablonka *et al.* 1995; Lachmann & Jablonka 1996; Shea *et al.* 2011; Geoghegan & Spencer 2012, 2013). Despite their important evolutionary implications, however, these models and expectations do not seem to have been explored empirically to date for any natural system. Our results for *H. foetidus* provide compelling evidence that transmissibility is an individually variable trait, possibly determined genetically, with consequences for individual fitness and subject to phenotypic selection. Directional phenotypic selection on transmissibility was likely a correlated outcome of selection on seed size variability. The sign of phenotypic selection on transmissibility varied between populations, and population differences in mean transmissibility were consistent with the contrasting signs of the directional selection at each site. These findings support the notion that (Darwinian) natural selection may drive adaptive evolution of epigenetic transmissibility, but with cascading implications for epigenetically driven evolution of acquired characters in a (neo-)Lamarckian way

(Jablonka & Lamb 1995; Jablonka 2013). For example, the seedling recruitment advantage accrued to plants with lower transmissibility in the NAV and PLL sites will enhance the opportunities for both the 'molecular exploration' of the environment (Jorgensen 2011; Jablonka 2013) and the selective screening of epigenetically induced seedling traits, independently of their genetic differences. The reverse is expected to apply at the low-elevation TEJ site, where current selection for increased transmissibility may narrow the 'exploration space' of the offspring and limit opportunities for selection and short-term adaptive responses based on epigenetic variation. These hypothesized differences between populations in epigenetic evolutionary potential could explain why populations of *H. foetidus* have low seedling recruitment and are declining at lower elevations, while recruitment is high and populations are expanding at high elevations (this study and C. M. Herrera and M. Medrano, Unpublished), since epigenetic diversity may endow populations with enhanced colonizing ability, expanding potential and resistance to perturbations (Richards *et al.* 2012; Latzel *et al.* 2013).

Concluding remarks

This study illustrates the potential of observational approaches combining ecological, genetic and epigenetic information as a means to explore integrative hypotheses and suggest experiments in the emerging fields of population epigenetics and ecological epigenetics (Bossdorf *et al.* 2008; Richards 2008). Results emphasize the importance of a population perspective to achieve a realistic understanding of the entangled relationships between ecological, genetic and epigenetic effects in natural plant populations (Richards 2008; Herrera & Bazaga 2010, 2011). In particular, this investigation calls attention to the significance of treating epigenetic and genetic variation as two possibly intertwined evolutionary factors acting simultaneously in natural populations (Herrera & Bazaga 2011), an aspect that is inevitably missed in experiments that constrain genetic variation by design. After experimental studies have firmly established the role of heritable epigenetic variation as an autonomous source of phenotypic variation, the time is ripe to start exploring the possible creative synergies between genetic and epigenetic layers of variation in natural populations.

Acknowledgements

We thank Laura Cabral and Esmeralda López for laboratory assistance; Conchita Alonso for discussion; the University of Oslo Bioportal and Centro de Supercomputación de Galicia (CESGA) for access to computer resources; and two reviewers for cogent criticism that improved considerably the study. Permission to work in the Sierra de Cazorla was provided by the Consejería de Medio Ambiente, Junta de Andalucía. Work supported in part by Grant CGL2010-15964 (Ministerio de Ciencia e Innovación).

References

Banks JA, Fedoroff N (1989) Patterns of developmental and heritable change in methylation of the suppressor-mutator transposable element. *Developmental Genetics*, **10**, 425–437.

Becker C, Weigel D (2012) Epigenetic variation: origin and transgenerational inheritance. *Current Opinion in Plant Biology*, **15**, 562–567.

Berger F, Twell D (2011) Germline specification and function in plants. *Annual Review of Plant Biology*, **62**, 461–484.

Biere A (1991) Parental effects in *Lychnis flos-cuculi*. I: seed size, germination and seedling performance in a controlled environment. *Journal of Evolutionary Biology*, **3**, 447–465.

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

Caballero A, García-Pereira MJ, Quesada H (2013) Genomic distribution of AFLP markers relative to gene locations for different eukaryotic species. *BMC Genomics*, **14**, 528.

Castellanos MC, Medrano M, Herrera CM (2008) Genetic and environmental effects on subindividual variation in seed traits in a European *Aquilegia*. *Botany*, **86**, 1125–1132.

Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics*, **18**, 237–268.

Consortium MERPD, Aksoy S, Almeida-Val VMF *et al.* (2013) Permanent genetic resources added to Molecular Ecology Resources database 1 October 2012–30 November 2012. *Molecular Ecology Resources*, **13**, 341–343.

Crean AJ, Marshall DJ (2009) Coping with environmental uncertainty: dynamic bet hedging as a maternal effect. *Philosophical Transactions of the Royal Society. Biological Sciences*, **364**, 1087–1096.

Das OP, Messing J (1994) Variegated phenotype and developmental methylation changes of a maize allele originating from epimutation. *Genetics*, **136**, 1121–1141.

Dussle CM, Quint M, Melchinger AE, Xu ML, Lubberstedt T (2003) Saturation of two chromosome regions conferring resistance to SCMV with SSR and AFLP markers by targeted BSA. *Theoretical and Applied Genetics*, **106**, 485–493.

Endler JA (1986) *Natural Selection in the Wild*. Princeton University Press, Princeton, New Jersey.

Feinberg AP, Irizarry RA (2010) Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 1757–1764.

Garrido JL, Rey PJ, Herrera CM (2005) Pre- and post-germination determinants of the spatial variation in recruitment in the perennial herb *Helleborus foetidus* L. (Ranunculaceae). *Journal of Ecology*, **93**, 60–66.

Garrido JL, Rey PJ, Herrera CM (2007) Regional and local variation in seedling emergence, mortality and recruitment of a perennial herb in Mediterranean mountain habitats. *Plant Ecology*, **190**, 109–121.

Geoghegan JL, Spencer HG (2012) Population-epigenetic models of selection. *Theoretical Population Biology*, **81**, 232–242.

Geoghegan JL, Spencer HG (2013) Exploring epiallele stability in a population-epigenetic model. *Theoretical Population Biology*, **83**, 136–144.

Goll MG, Bestor TH (2005) Eukaryotic cytosine methyltransferases. *Annual Review of Biochemistry*, **74**, 481–514.

Gutierrez-Marcos JF, Dickinson HG (2012) Epigenetic reprogramming in plant reproductive lineages. *Plant and Cell Physiology*, **53**, 817–823.

Harper JL (1977) *Population Biology of Plants*. Academic Press, London, UK.

Herrera CM (2009) *Multiplicity in Unity. Plant Subindividual Variation and Interactions with Animals*. University of Chicago Press, Chicago, Illinois.

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, Medrano M, Bazaga P (2013) Epigenetic differentiation persists after male gametogenesis in natural populations of the perennial herb *Helleborus foetidus* (Ranunculaceae). *PLoS One*, **8**, e70730.

Herselman L, Thwaites R, Kimmins FM, Courtois B, van der Merwe PJA, Seal SE (2004) Identification and mapping of AFLP markers linked to peanut (*Arachis hypogaea* L.) resistance to the aphid vector of groundnut rosette disease. *Theoretical and Applied Genetics*, **109**, 1426–1433.

Jablonka E (2013) Epigenetic inheritance and plasticity: the responsive germline. *Progress in Biophysics and Molecular Biology*, **111**, 99–107.

Jablonka E, Lamb MJ (1995) *Epigenetic Inheritance and Evolution. The Lamarckian Dimension*. Oxford University Press, Oxford, UK.

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

Jablonka E, Oborny B, Molnár I, Kisdi E, Hofbauer J, Czaran T (1995) The adaptive advantage of phenotypic memory in changing environments. *Philosophical Transactions of the Royal Society. Biological Sciences*, **350**, 133–141.

Jamieson A, Taylor SS (1997) Comparisons of three probability formulae for parentage exclusion. *Animal Genetics*, **28**, 397–400.

Johannes F, Porcher E, Teixeira FK *et al*. (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.

Kesavan M, Song JT, Seo HS (2013) Seed size: a priority trait in cereal crops. *Physiologia Plantarum*, **147**, 113–120.

Kingsolver JG, Pfennig DW (2007) Patterns and power of phenotypic selection in nature. *BioScience*, **57**, 561–572.

Köhler C, Makarevich G (2006) Epigenetic mechanisms governing seed development in plants. *EMBO Reports*, **7**, 1223–1227.

Lachmann M, Jablonka E (1996) The inheritance of phenotypes: an adaptation to fluctuating environments. *Journal of Theoretical Biology*, **181**, 1–9.

Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. *Evolution*, **37**, 1210–1226.

Latzel V, Allan E, Silveira AB, Colot V, Fischer M, Bossdorf O (2013) Epigenetic diversity increases the productivity and stability of plant populations. *Nature Communications*, **4**, 2875.

Lira-Medeiros CF, Parisod C, Fernandes RA, Mata CS, Cardoso MA, Ferreira PCG (2010) Epigenetic variation in mangrove plants occurring in contrasting natural environment. *PLoS One*, **5**, e10326.

Manasse RS, Stanton ML (1991) The influence of the mating system on seed size variation in *Crinum erubescens* (Amaryllidaceae). *Evolution*, **45**, 883–890.

Manzaneda AJ, Rey PJ (2008) Geographic variation in seed removal of a myrmecochorous herb: influence of variation in functional guild and species composition of the disperser assemblage through spatial and temporal scales. *Ecography*, **31**, 583–591.

McClintock B (1950) The origin and behavior of mutable loci in maize. *Proceedings of the National Academy of Sciences of the United States of America*, **36**, 344–355.

Migicovsky Z, Kovalchuk I (2012) Epigenetic modifications during angiosperm gametogenesis. *Frontiers in Plant Science*, **3**, 20.

North H, Baud S, Debeaujon I *et al*. (2010) Arabidopsis seed secrets unravelled after a decade of genetic and omics-driven research. *The Plant Journal*, **61**, 971–981.

Pérez-Figueroa A (2013) MSAP: a tool for the statistical analysis of methylation-sensitive amplified polymorphism data. *Molecular Ecology Resources*, **13**, 522–527.

Philippi T, Seger J (1989) Hedging one's evolutionary bets, revisited. *Trends in Ecology & Evolution*, **4**, 41–44.

Platt A, Vilhjalmsson BJ, Nordborg M (2010) Conditions under which genome-wide association studies will be positively misleading. *Genetics*, **186**, 1045–1052.

Price AL, Zaitlen NA, Reich D, Patterson N (2010) New approaches to population stratification in genome-wide association studies. *Nature Reviews Genetics*, **11**, 459–463.

R Development Core Team (2012) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.

Raman H, Moroni JS, Sato K, Read BJ, Scott BJ (2002) Identification of AFLP and microsatellite markers linked with an aluminium tolerance gene in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics*, **105**, 458–464.

Ramírez JM, Rey PJ, Alcántara JM, Sánchez-Lafuente AM (2006) Altitude and woody cover control recruitment of *Helleborus foetidus* in a Mediterranean mountain area. *Ecography*, **29**, 375–384.

Rando OJ, Verstrepen KJ (2007) Timescales of genetic and epigenetic inheritance. *Cell*, **128**, 655–668.

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

Richards EJ (2008) Population epigenetics. *Current Opinion in Genetics & Development*, **18**, 221–226.

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

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

Richards CL, Schrey AW, Pigliucci M (2012) Invasion of diverse habitats by few Japanese knotweed genotypes is

correlated with epigenetic differentiation. *Ecology Letters*, **15**, 1016–1025.
Saze H, Scheid OM, Paszkowski J (2003) Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nature Genetics*, **34**, 65–69.
Schmitz RJ, Schultz MD, Lewsey MG *et al.* (2011) Transgenerational epigenetic instability is a source of novel methylation variants. *Science*, **334**, 369–373.
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.
Shea N, Pen I, Uller T (2011) Three epigenetic information channels and their different roles in evolution. *Journal of Evolutionary Biology*, **24**, 1178–1187.
Silvertown J (1989) The paradox of seed size and adaptation. *Trends in Ecology & Evolution*, **4**, 24–26.
Simons AM, Johnston MO (1997) Developmental instability as a bet-hedging strategy. *Oikos*, **80**, 401–406.
Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 9440–9445.
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, van Gurp TP (2012) Transgenerational effects of stress exposure on offspring phenotypes in apomictic dandelion. *PLoS One*, **7**, e38605.
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.
Xiao WY, Brown RC, Lemmon BE, Harada JJ, Goldberg RB, Fischer RL (2006) Regulation of seed size by hypomethylation of maternal and paternal genomes. *Plant Physiology*, **142**, 1160–1168.

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

Data accessibility

MSAP, AFLP, microsatellite, seed mass and seedling data used in this study deposited at DRYAD: doi:10.5061/dryad.s04v1.

Supporting information

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

Appendix S1 Genetic structure of plants sampled for the study.

Fig. S1 Relationships between methylation transmissibility and the four significantly associated AFLP loci.

Fig. S2 Variation between plants and populations in mean and variance of individual seed mass distributions.