American Journal of Botany

RESEARCH ARTICLE

Transgenerational epigenetics: Inheritance of global cytosine methylation and methylation-related epigenetic markers in the shrub *Lavandula latifolia*

Carlos M. Herrera[1,3], Conchita Alonso[1], Mónica Medrano[1], Ricardo Pérez[2], and Pilar Bazaga[1]

Manuscript received 16 August 2017; revision accepted 22 January 2018.

[1] Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Américo Vespucio 26, 41092 Sevilla, Spain

[2] Instituto de Investigaciones Químicas, Centro de Investigaciones Científicas Isla de La Cartuja, Consejo Superior de Investigaciones Científicas (CSIC)–Universidad de Sevilla, Sevilla, Spain

[3] Author for correspondence (e-mail: herrera@ebd.csic.es)

Citation: Herrera, C. M., C. Alonso, M. Medrano, R. Pérez, and P. Bazaga. 2018. Transgenerational epigenetics: Inheritance of global cytosine methylation and methylation-related epigenetic markers in the shrub *Lavandula latifolia*. *American Journal of Botany* 105(4): 1–8.

doi:10.1002/ajb2.1074

PREMISE OF THE STUDY: The ecological and evolutionary significance of natural epigenetic variation (i.e., not based on DNA sequence variants) variation will depend critically on whether epigenetic states are transmitted from parents to offspring, but little is known on epigenetic inheritance in nonmodel plants.

METHODS: We present a quantitative analysis of transgenerational transmission of global DNA cytosine methylation (= proportion of all genomic cytosines that are methylated) and individual epigenetic markers (= methylation status of anonymous MSAP markers) in the shrub *Lavandula latifolia*. Methods based on parent-offspring correlations and parental variance component estimation were applied to epigenetic features of field-growing plants ('maternal parents') and greenhouse-grown progenies. Transmission of genetic markers (AFLP) was also assessed for reference.

KEY RESULTS: Maternal parents differed significantly in global DNA cytosine methylation (range = 21.7–36.7%). Greenhouse-grown maternal families differed significantly in global methylation, and their differences were significantly related to maternal origin. Methylation-sensitive amplified polymorphism (MSAP) markers exhibited significant transgenerational transmission, as denoted by significant maternal variance component of marker scores in greenhouse families and significant mother-offspring correlations of marker scores.

CONCLUSIONS: Although transmission-related measurements for global methylation and MSAP markers were quantitatively lower than those for AFLP markers taken as reference, this study has revealed extensive transgenerational transmission of genome-wide global cytosine methylation and anonymous epigenetic markers in *L. latifolia*. Similarity of results for global cytosine methylation and epigenetic markers lends robustness to this conclusion, and stresses the value of considering both types of information in epigenetic studies of nonmodel plants.

KEY WORDS amplified fragment length polymorphism (AFLP); epigenetic variation; global cytosine methylation; inheritance; Lamiaceae; methylation-sensitive amplified polymorphism (MSAP).

Natural epigenetic variation (i.e., not based on DNA sequence variants) has been recently related to phenotypic and functional diversity in plants (Medrano et al., 2014; Ci et al., 2015; Hu et al., 2015; Kooke et al., 2015; Wilschut et al., 2016). The ecological and evolutionary significance of such epigenetic variation will depend critically on whether epigenetic states that are related to organismal features are transmitted from parents to offspring (i.e., epigenetic states are not completely reset between generations; Jablonka and Raz, 2009; Jablonka and Lamb, 2015; Uller et al., 2015; Kronholm and Collins, 2016), a situation that has been reported frequently by recent investigations (Cortijo et al., 2014; Miska and Ferguson-Smith, 2016; Quadrana and Colot, 2016; Zheng et al., 2017). In contrast to developmental epigenetics, transgenerational epigenetics involves heritable changes in DNA methylation (see Quadrana and Colot, 2016, for review). Although it has been sometimes advocated that the concept of transgenerational epigenetic inheritance should

be restricted to cases where epigenetic changes remain unaltered for a number of successive generations (e.g., Pecinka and Mittelsten Scheid, 2012), recent usage tends to be less restrictive and the concept can also be applied in situations where epigenetic states are not reset between two consecutive generations (Quadrana and Colot, 2016). We adhere to this more liberal usage in this paper.

Available evidence of transgenerational epigenetic transmission in plants refers nearly exclusively to a handful of crop and model species with detailed genomic information available (see reviews in Hauser et al., 2011; Quadrana and Colot, 2016). Remarkably few attempts have been done so far at quantitatively assessing epigenetic inheritance in nonmodel species without a reference genome (but see Herrera et al., 2013; Avramidou et al., 2015). Broadening the phylogenetic scope of studies on transgenerational epigenetics (sensu Quadrana and Colot, 2016) beyond the narrow domain of model plants will help to evaluate the generality of the phenomenon and contribute to a better understanding of the importance of epigenetic mechanisms in plants, as recently emphasized by Rensing (2017) in a related context. DNA cytosine methylation is a key mechanism for epigenetic regulation in plants (Grant-Downton and Dickinson, 2005, 2006; Gehring and Henikoff, 2007) whose magnitude, genome-wide patterns, and functional aspects have a significant phylogenetic component and vary across plant lineages (Zemach et al., 2010; Alonso et al., 2016; Vidalis et al., 2016). Phylogenetically restricted and/or biased species sampling might therefore lead to distorted or biased views on the prevalence of epigenetic inheritance in plants as a whole.

We present in this paper a quantitative analysis of transgenerational transmission of global DNA cytosine methylation (= proportion of all genomic cytosines that are methylated; also sometimes termed "bulk methylation" or "genome-wide methylation") and individual epigenetic markers (= methylation status of anonymous genomic markers) in the shrub *Lavandula latifolia*. Although data on global cytosine methylation are not informative on the genomic positions where methylation occurs, its analysis is helpful to evaluate the overall importance of this epigenetic mark in nonmodel organisms without detailed genomic information (Rozhon et al., 2008; Alonso et al., 2016). Furthermore, variation in global cytosine methylation is correlated with changes in the methylation status of specific genic and intergenic regions, and is consequential for gene expression, genomic instability (Steward et al., 2002; Baubec et al., 2009; Bonchev and Parisod, 2013; Vidalis et al., 2016), and organismal features (Sano et al., 1990; Tatra et al., 2000; Kondo et al. 2006). Anonymous epigenetic markers, on the other hand, provide information on the genome-wide patterns of cytosine methylation, and their variation across individuals or populations is often related to differences in functional traits (Medrano et al., 2014; Herrera et al., 2017). Taken together, global cytosine methylation and anonymous epigenetic markers thus provide complementary views of genome-wide epigenetic characteristics of individuals, and have been often applied in ecological epigenetics studies of nonmodel plants (Schulz et al., 2013; Alonso et al., 2014, 2016, 2018; Wilschut et al., 2016).

Epigenetic characteristics will be treated here as individual phenotypic traits, and standard methods commonly used in quantitative genetics studies of trait heritability, namely parent-offspring correlations and parental variance component estimation in the progeny (Roff, 1997; Lynch and Walsh, 1998), will be applied. Some peculiarities of epigenetic variation, notably its potential lability in response to the environment, call for extended quantitative genetic models and more complex experimental and analytical designs (Gorelick, 2005; Gorelick and Laubichler, 2008). For this reason, we will not attempt here to formally relate our quantitative results to the notion of 'heritability'. In addition to documenting extensive inheritance of the epigenetic features considered in *Lavandula latifolia*, the present study also aims to illustrate a simple analytical approach that can be easily implemented experimentally to test and quantify the transgenerational transmission of anonymous epigenetic markers in nonmodel plants. In order to obtain a reference level against which to compare the transmission of molecular epigenetic characteristics, we will also concurrently assess transmission of anonymous genetic markers using the same experimental and analytical methods.

MATERIALS AND METHODS

Study species

Lavandula latifolia (Lamiaceae) is a long-lived, low evergreen shrub characteristic of clearings and well-lit undergrowth in open woodlands of the eastern Iberian Peninsula at 1000–1600 m a.s.l. Flowering takes place during July–October. Flowers are hermaphrodite, self-compatible, and insect pollinated. The species reproduces exclusively by seeds, which are small (~1 mg), lack special mechanisms for dispersal, and ripen by the end of summer (see, e.g., Herrera and Bazaga, 2016, for further information on the ecology and natural history of the species).

Field and greenhouse

Material for this study was collected from a large population of *L. latifolia* located in Cuevas Bermejas, Sierra de Cazorla (37.96523°N, 2.84981°W, 1180 m elevation; Jaén Province, southeastern Spain). Fifteen adult plants of approximately similar size were sampled along a 125-m linear transect ('maternal parents' hereafter). Samples of first-year leaves and newly ripe seeds were collected from each plant in October 2005. Leaves were placed in paper envelopes and dried immediately at ambient temperature in sealed containers with silica gel. Seeds were kept in paper envelopes at ambient temperature until sown in a greenhouse about one month later. Seeds germinated within 2–3 weeks after sowing, and the resulting plants were grown individually in flowerpots with similar soil mixture (75% peat moss, 25% vermiculite), light (natural daylight), temperature (~27°C) and air humidity (~45%), and were watered as needed. Over the course of the experiment, plants were transplanted twice to larger flowerpots. When they were 8-month-old juveniles, leaf samples were collected from all the plants that remained alive, and were dried immediately at ambient temperature in containers with silica gel.

Laboratory

Total genomic DNA was extracted from all field and greenhouse leaf samples using Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and the manufacturer's protocol. Global DNA cytosine methylation analyses, and genetic and epigenetic fingerprinting, were conducted on these DNA samples.

Global DNA cytosine methylation was determined for each sample using the chromatographic technique described in detail by Alonso et al. (2014, 2016). DNA was digested with DNA Degradase PlusTM (Zymo Research, Irvine, CA), a nuclease mix

that degrades DNA to its individual nucleoside components. To allow for testing the statistical significance of individual variation between field-sampled plants in DNA methylation level, 2–4 independent technical replicates of DNA hydrolyzate were prepared for each individual. DNA cytosine methylation was determined on these samples by reversed phase high performance liquid chromatography (HPLC) with spectrofluorimetric detection. Global cytosine methylation was estimated as 100 × 5mdC/(5mdC + dC), where 5mdC and dC are the integrated areas under the peaks for 5-methyl-2′-deoxycytidine and 2′-deoxycytidine, respectively. The position of each nucleoside was determined using commercially available standards (Sigma Aldrich, St. Louis, Missouri, USA).

Maternal parents and greenhouse plants were genetically fingerprinted using amplified fragment length polymorphism (AFLP) markers. The AFLP analysis was performed using standard protocols involving the use of fluorescent dye-labeled selective primers. Each plant was fingerprinted using three different EcoRI + 3 / MseI + 3 primer pair combinations. Fragment separation and detection was made using an ABI PRISM 3130xl DNA sequencer (Applied Biosystems, Foster City, California, USA), and the presence or absence of each AFLP fragment in each individual plant was scored manually by visualizing electropherograms with GeneMapper 3.7 software (Applied Biosystems, Foster City, California, USA). Only fragments ≥150 base pairs in size were considered to reduce possible biases arising from size homoplasy (Vekemans et al., 2002). A total of 132 AFLP markers were obtained for the combined sample of offspring and maternal parents, 89 of which were polymorphic (≥2% of samples with variant scores). Only these polymorphic markers will be considered in all analyses.

Plants were characterized epigenetically using the methylation-sensitive amplified polymorphism technique (MSAP; Schulz et al., 2013; Guevara et al., 2017). Despite some acknowledged limitations (Schrey et al., 2013; Fulneček and Kovařik, 2014; Alonso et al., 2016), anonymous MSAP markers are useful to investigate variation in genome-wide patterns of cytosine methylation in non-model plants lacking detailed genomic information (Medrano et al., 2014; Foust et al., 2016; Herrera et al., 2016; Wilschut et al., 2016). MSAP is a modification of the standard AFLP technique that uses the methylation-sensitive restriction enzymes HpaII and MspI in parallel runs in combination with another restriction enzyme. The isoschizomers HpaII and MspI recognize the same tetranucleotide 5′-CCGG but have differential sensitivity to methylation at the inner or outer cytosine. Differences in the products obtained with HpaII and MspI thus reflect different methylation states at the cytosines of the CCGG sites recognized by HpaII or MspI cleavage sites (Schulz et al., 2013; Fulneček and Kovařic, 2014). The MSAP assays for this study were conducted using four HpaII-MspI + 2 / MseI + 3 primer combinations. Fragment separation and detection were made using an ABI PRISM 3130xl DNA sequencer, and the presence or absence of HpaII/MseI and MspI/MseI fragments in each sample was scored manually by visualizing electropherograms with GeneMapper 3.7 software.

Data analysis

The 'Mixed Scoring 2' transformation scheme recommended by Schulz et al. (2013) was used to obtain MSAP markers from the two presence-absence matrices for MSAP fragments obtained with the four HpaII-MseI and MspI-MseI primer combination pairs. MSAP fragments were transformed into three distinct sets of markers, corresponding to the *h*, *m*, and *u* (= unmethylated) types in the Schulz et al. (2013) terminology. These three marker types corresponded to 1/0, 0/1, and 1/1 HpaII/MspI banding patterns, respectively (Conditions III, II, and I of Schulz et al. 2013, respectively, where further details and rationale are presented). The *h* and *m* types reflected ^{HMe}CCG methylation (external hemimethylation) and ^{HMe}CG + ^{Me}CG methylation (internal methylation plus hemimethylation), respectively (see Schulz et al., 2013, for full details; but see Fulneček and Kovařik, 2014). The function Extract_MSAP_epigenotypes from Schulz et al. (2013) was used to fingerprint all field-sampled and greenhouse plants with these three MSAP marker types. A total of 306 MSAP markers were obtained in the combined sample of offspring and maternal parents, 197 of which were polymorphic and will be considered in the analyses.

Transmission from maternal parents to progeny of global cytosine methylation and genetic (AFLP) and epigenetic (MSAP) markers was assessed by testing the statistical significance of (a) maternal variance components in the greenhouse-grown progeny and (b) mother-offspring correlations. For global cytosine methylation, the variance component accounted for by maternal parents was obtained by fitting a linear mixed model to the progeny data with maternal family as the single random effect. Statistical significance of the maternal variance component was obtained by random permutations of individuals among families. For presence-absence, binary AFLP and MSAP markers, the maternal variance component in the progeny was obtained by fitting a generalized linear mixed model for each marker using the binomial error distribution and 'logit' link function, and then computing the Bayesian R^2 for the fitted model using the formula proposed by Davies et al. (2015; see also Vazquez et al., 2009). Values obtained for *all* markers of each type were then averaged, and statistical significance of the departure of these means from zero was assessed by comparing observed values with distributions obtained by random permutations of individuals among families.

For global cytosine methylation, the mother-offspring linear correlation was computed between maternal values (= mean of 2–4 replicates per plant) and the corresponding offspring means for the 13 plants with paired mother-offspring data available. This small number of data points and the presence of one putative influential point (assessed by considering Studentized residuals and Cook's distances) could bias tests of statistical significance based on asymptotically-derived *P*-values. To account for this possibility, statistical significance of the mother-offspring correlation for global cytosine methylation was tested by random permutations. Furthermore, the mother-offspring regression was fitted using robust regression, which minimizes the effect of outliers and leverage points on regression parameter estimates (Rousseeuw and Leroy, 1987). For binary AFLP and MSAP marker data, mother-offspring correlations were obtained separately for each marker as the phi coefficient (= mean square contingency coefficient) of the two-way contingency table of marker scores (present-absent) × generation (mother-offspring). The phi coefficient is a special case of the Pearson product-moment correlation coefficient for two dichotomous variables (Everitt, 2006). Values obtained for individual markers were then averaged for each marker class. The statistical significance of the means thus obtained was assessed by comparisons with distributions obtained by random permutations of progenies among maternal parents.

All statistical analyses were carried out using the R environment (R Development Core Team, 2017). The 'lmer' and 'glmer' functions

from the lme4 package were used to fit linear mixed and generalized linear mixed models, respectively (Bates et al., 2015). Phi coefficients were computed with the phi function from the 'psych' package (Revelle 2017).

RESULTS

Global cytosine methylation

Global DNA cytosine methylation of adult *L. latifolia* shrubs sampled in the field ranged between 21.7–36.7% of total cytosines (Fig. 1). These individual differences were statistically significant ($F_{14,33} = 4.23$, $P = 0.0003$). Between-plant variation in global methylation was related to spatial location, as denoted by the statistically significant rank correlation between global methylation and position along the sampling transect ($r_w = 0.534$, $P = 0.021$; Spearman rank correlation coefficient weighted by number of replicates per estimate).

Greenhouse-grown maternal families from field-collected seeds differed significantly in global methylation ($F_{12,90} = 2.34$, $P = 0.012$), and these differences were related to their maternal origin. The proportion of total sample variance of global methylation in the progeny accounted for by maternal origin was 0.152, which was significantly greater than zero ($P = 0.011$, randomization test with 10^4 permutations). The range of family means (23.7–29.9% of total cytosines) was substantially narrower than the range exhibited by maternal parents (Fig. 2), but there was relatively little disparity in overall means for the two generations (28.7% and 26.0%, for parents and progenies, respectively). There was a positive, statistically significant linear relationship between global methylation of maternal parents and the mean global methylation of their respective progeny (Fig. 3) ($r = 0.540$, $P = 0.030$, randomization test with 10^4 permutations).

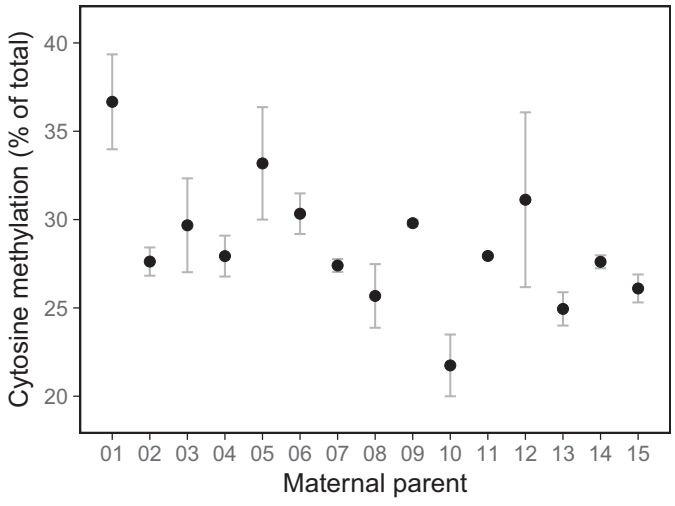

FIGURE 1. Variation in global DNA cytosine methylation among the $N = 15$ wild-growing, adult *Lavandula latifolia* plants sampled. These plants were the maternal parents of half-sib families grown in the greenhouse. Dots represent the mean value for the set of technical replicates analyzed for each plant, and vertical segments extend over ± 1 SE around the mean. The sequence of plant names reflects their position along the sampling transect.

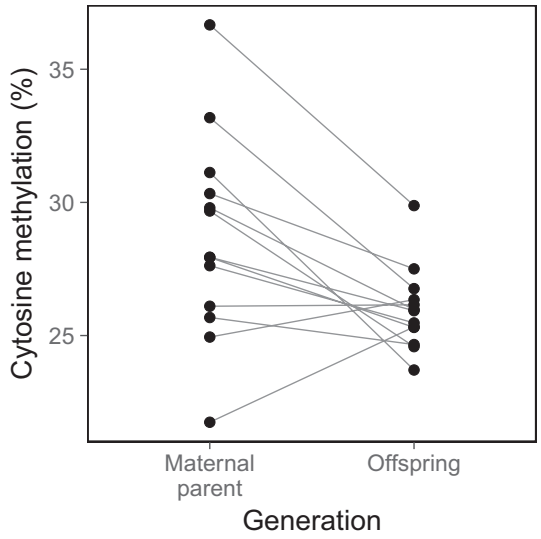

FIGURE 2. Mean values of global cytosine methylation of wild maternal parents and their respective progenies grown in the greenhouse from field-collected seeds ($N = 13$, because there were two maternal parents without seedlings).

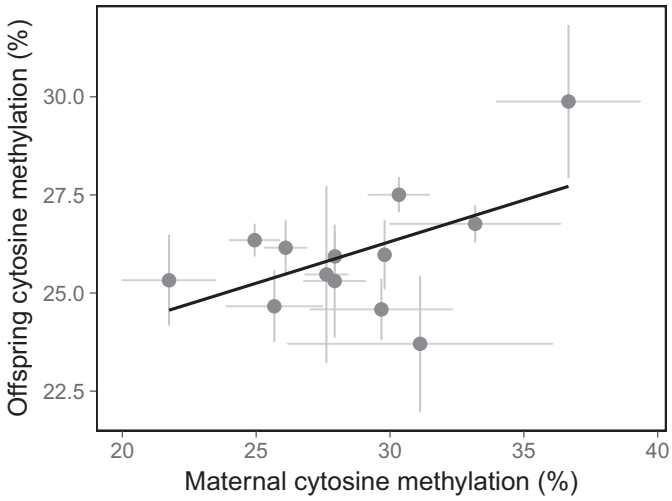

FIGURE 3. Relationship between mean global DNA cytosine methylation of half-sib maternal families grown in the greenhouse, and the corresponding value for their respective maternal parents growing in the wild. Dots represent mean values for technical replicates (maternal plants) or individuals (families), and segments extend over ± 1 SE around the mean. The robust regression fitted to the means is shown ($y = 19.962 + 0.212\,x$; $R^2 = 0.275$).

Genetic and epigenetic markers

Both genetic (AFLP) and epigenetic (MSAP) markers exhibited transgenerational transmission, as denoted by the combination of significant maternal variance component of marker scores in the greenhouse families and significant mother-offspring correlations of marker scores (Table 1). These two magnitudes were larger for AFLP than for MSAP markers (variance components 0.31 vs. 0.20; mother-offspring correlations, 0.29 vs. 0.15; for AFLP and MSAP, respectively, Table 1), the differences being statistically significant

TABLE 1. Proportion of total variance explained by differences between maternal families, and mother-offspring correlations (phi coefficient), for genetic (AFLP) and epigenetic (MSAP) marker scores in plants of *Lavandula latifolia* grown in the greenhouse from field-collected seeds.

		MSAP			
	AFLP	**All**	***h*-type**	***m*-type**	***u*-type**
Number of polymorphic markers	89	197	105	49	43
Proportion of variance between maternal families:					
Mean ± SE	0.312 ± 0.034	0.198 ± 0.022	0.198 ± 0.031	0.150 ± 0.039	0.255 ± 0.050
P-value	< 0.001	< 0.001	< 0.001	0.079	0.017
Mother-offspring correlation:					
Mean ± SE	0.289 ± 0.017	0.145 ± 0.008	0.132 ± 0.011	0.158 ± 0.017	0.161 ± 0.019
P-value	< 0.001	0.0039	0.013	0.29	0.032

Notes: A marker was considered polymorphic if ≥2% of samples showed a variant score.

(χ-squared = 15.87 and 17.70, df = 1, $P < 0.0001$, Kruskal-Wallis rank sum tests for maternal variance component and mother-offspring correlations, respectively).

There were differences between the three MSAP marker types recognized here in transmission-related parameters. Maternal variance component in the progeny and mother-offspring correlations were significantly greater than zero for *h*- and *u*-type markers, but not for *m*-type markers (Table 1).

DISCUSSION

Cytosine methylation is a major mechanism for epigenetic modification of DNA in plants that is involved in regulation of gene expression, control of genomic integrity, and plant growth and development (Richards, 1997; Grant-Downton and Dickinson 2005, 2006). Methylated cytosines occur throughout genic and intergenic spaces of nuclear plant genomes (Cokus et al., 2008; Lister et al., 2008), hence intraspecific variation in global cytosine methylation reflects changes in the methylation status of specific genic and intergenic regions, and has functional consequences in terms of altered gene expression and genomic instability (Messeguer et al., 1991; Steward et al., 2002; Baubec et al., 2009; Vidalis et al., 2016). Therefore, as noted in the introductory material of this article, although global cytosine methylation measurements do not provide information on the genomic locations at which methylation occurs, they provide a technically feasible avenue to explore genome-wide epigenetic variation in nonmodel species without a reference genome (Alonso et al., 2016). Little is known so far, however, on natural levels of variation in global cytosine methylation in wild plant populations. In a previous study, Alonso et al. (2014) found that global cytosine methylation differed significantly among individuals of the perennial herb *Helleborus foetidus*, ranging between 26–36%. This variability level is comparable to the 22–37% range found here for adult plants of *L. latifolia* sampled in the field, whose individual differences were also statistically significant. Our results thus corroborate that intraspecific variation in global cytosine methylation can sometimes be as broad or broader than differences between species (Alonso et al., 2014, 2015).

Considerable experimental evidence indicates that alterations in genomic methylation levels follow plant exposure to biotic or abiotic agents (e.g., pathogens, herbivores, low temperatures, water stress; Steward et al., 2002; Lukens and Zhang, 2007; Verhoeven et al., 2010; Grativol et al., 2012). Differential exposure of adult *L. latifolia* shrubs in the field during their lifetimes to stressing agents could thus have contributed to generate the broad individual heterogeneity observed in cytosine methylation levels, as proposed by Alonso et al. (2014) for *H. foetidus*. This view is consistent with (1) the narrower range of methylation levels exhibited by the progeny grown under homogeneous greenhouse conditions relative to maternal parents; and (2) the significant relationship found between cytosine methylation level and plant position along the sampling gradient, because it seems reasonable to expect that spatially closer plants should experience more similar environments than plants farther apart. It cannot be ruled out, however, that spatial distance and genetic relatedness were correlated across sampled plants (Herrera and Bazaga, 2016), and that observed individual differences in methylation level were in part the consequence of different genotypes reacting to the same environmental variables in different epigenetic ways. In any case, significant mother-offspring correlation and significant maternal variance component in the progeny clearly show that global cytosine methylation levels were only incompletely reset across generations, thus revealing the transgenerationally heritable nature of the trait in this species.

Dominant genetic markers do not allow unequivocal inference of individual genotypes, hence presence-absence scores for AFLP and its methylation-sensitive variant MSAP markers are best treated as phenotypic traits as is done here (Bonin et al., 2007). Obtaining quantitative estimates of the extent of transgenerational transmission for binary phenotypic traits, however, is not exempt from conceptual and analytical difficulties (Villemereuil et al., 2013). The approach adopted here included extensions of variance partitioning methods to generalized linear mixed models (Vazquez et al., 2009; Davies et al., 2015) and the application of phi coefficients as a statistical equivalent to the Pearson correlation coefficient suitable for binary data. Used in combination with permutation tests, these methods have proven useful to demonstrate significant parent-offspring correlations, significant maternal variance components and, consequently, significant inheritance of both genetic (AFLP) and epigenetic (MSAP) dominant markers in *L. latifolia*. Significant inheritance of MSAP markers was previously inferred by Herrera et al. (2013, 2014) for the perennial herb *Helleborus foetidus* on the basis of high sporophyte-to-gametophyte transmissibility (see also Avramidou et al., 2015). Significant inheritance of AFLP markers corroborated a priori expectations based on the long-known fact that many of them segregate as stable Mendelian loci (Maughan et al., 1996; Castiglioni et al., 1999; Lerceteau and Szmidt, 1999). Transmission-related parameters for AFLP markers (parent-offspring correlation and maternal variance component) thus provided a useful reference level for comparison with those obtained for MSAP markers.

Although statistically significant, the magnitudes of transmission-related parameters for MSAP markers considered

collectively were only about 63% (maternal variance component) and 50% (mother-offspring correlation) of corresponding figures for AFLP markers, which denotes a lower average transmission rate of epigenetic markers relative to genetic ones (see also Avramidou et al., 2015). This transgenerational epigenetic instability most likely reflects a combination of partial resetting of epigenomes at the sporophyte-to-gametophyte transition, and spontaneous forward-backward epimutations at CG sites, as reported for some model plants (Schmitz et al., 2011; Kawashima and Berger, 2014; van der Graaf et al., 2015; Quadrana and Colot, 2016). When considered separately, the three MSAP marker classes recognized here under the Schulz et al. (2013) 'Mixed Scoring 2' scheme differed in their transmission parameters, which were statistically significant for *h*- and *u*-type markers, but not for *m*-type ones. These differences could be related to heterogeneity among marker types in resetting frequency and/or epimutation rate due to differences in prevailing methylation status (*u*-type vs. the rest) or maintenance mechanisms of cytosine methylation in the CHG and CG contexts (Cokus et al., 2008; Lister et al., 2008; Osabe et al., 2014) with which *h*- and *m*-type markers are presumably associated, respectively (Schulz et al., 2013; but see Fulneček and Kovařik, 2014). Regardless of the actual mechanism underlying these differences, however, heterogeneity between marker types in transmission parameters found here provide additional justification for treating them separately in ecological epigenetics studies of nonmodel plants (Schulz et al., 2013). In particular, differences between marker types in extent of transgenerational transmission can help to interpret their differences in spatial structure, phenotypic correlates and relationships to habitat features in wild plant populations (Schulz et al., 2014; Medrano et al., 2014; Herrera and Bazaga, 2016; Herrera et al., 2016, 2017).

CONCLUSIONS

Theoretical arguments on the ecological and evolutionary significance of natural epigenetic variation have consistently highlighted the crucial importance of transgenerational stability of epigenetic variants (Shea et al., 2011; Geoghegan and Spencer, 2013; Furrow, 2014; Uller et al., 2015; Kronholm and Collins, 2016). Studies on a few crop and model species have revealed that the transmission rate of epigenetic states, albeit distinctly lower than that of genetic variants based on DNA sequences, can still be sufficiently high in some cases to sustain long-term selection responses (Cortijo et al., 2014; Lauria et al., 2014; van der Graaf et al., 2015; Quadrana and Colot, 2016). Although transmission of epigenetic markers was less faithful than that of genetic markers used as reference, the present study has revealed considerable transgenerational transmission of genome-wide global cytosine methylation and anonymous epigenetic markers in the nonmodel species *L. latifolia*. Similarity of results obtained for global cytosine methylation and epigenetic markers lends robustness to the conclusions of our study, and also stresses the value of considering both types of epigenetic information in studies of nonmodel plants without a reference genome (Alonso et al., 2016).

ACKNOWLEDGEMENTS

We are grateful to L. Cabral, M. García, and E. López for assistance in the greenhouse and laboratory; Consejería de Medio Ambiente, Junta de Andalucía, for permission to work in Sierra de Cazorla, granting access to greenhouse facilities at the San Jerónimo nursery, and assistance in maintaining plants; two anonymous reviewers for constructive criticisms; and Estación Biológica de Doñana-CSIC for contributing to greenhouse expenses. Partial financial support for this work was provided by grants CGL2013-43352-P and CGL2016-76605-P from the Spanish Ministerio de Economía y Competitividad.

DATA ACCESSIBILITY

The data sets used for this study, including global cytosine methylation estimates, and genetic and epigenetic fingerprints of maternal parents and their progenies, have been deposited at the public institutional repository of Consejo Superior de Investigaciones Científicas (Digital.CSIC), https://doi.org/10.20350/digitalcsic/8526.

LITERATURE CITED

Alonso, C., R. Pérez, P. Bazaga, and C. M. Herrera. 2015. Global DNA cytosine methylation as an evolving trait: phylogenetic signal and correlated evolution with genome size in Angiosperms. *Frontiers in Genetics* 6: 4.

Alonso, C., R. Pérez, P. Bazaga, M. Medrano, and C. M. Herrera. 2014. Individual variation in size and fecundity is correlated with differences in global DNA cytosine methylation in the perennial herb *Helleborus foetidus* (Ranunculaceae). *American Journal of Botany* 101: 1309–1313.

Alonso, C., R. Pérez, P. Bazaga, M. Medrano, and C. M. Herrera. 2016. MSAP markers and global cytosine methylation in plants: a literature survey and comparative analysis for a wild growing species. *Molecular Ecology Resources* 16: 80–90.

Alonso, C., R. Pérez, P. Bazaga, M. Medrano, and C. M. Herrera. 2018. Within-plant variation in seed size and inflorescence fecundity is associated with epigenetic mosaicism in the shrub *Lavandula latifolia* (Lamiaceae). *Annals of Botany* 121: 153–160.

Avramidou, E. V., A. G. Doulis, and F. A. Aravanopoulos. 2015. Determination of epigenetic inheritance, genetic inheritance, and estimation of genome DNA methylation in a full-sib family of *Cupressus sempervirens* L. *Gene* 562: 180–187.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.

Baubec, T., A. Pecinka, W. Rozhon, and O. Mittelsten Scheid. 2009. Effective, homogeneous and transient interference with cytosine methylation in plant genomic DNA by zebularine. *Plant Journal* 57: 542–554.

Bonchev, G., and C. Parisod. 2013. Transposable elements and microevolutionary changes in natural populations. *Molecular Ecology Resources* 13: 765–775.

Bonin, A., D. Ehrich, and S. Manel. 2007. Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. *Molecular Ecology* 16: 3737–3758.

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

Ci, D., Y. Song, Q. Du, M. Tian, S. Han, and D. Zhang. 2015. Variation in genomic methylation in natural populations of *Populus simonii* is associated with leaf shape and photosynthetic traits. *Journal of Experimental Botany* 67: 723–737.

Cokus, S. J., S. H. Feng, X. Y. Zhang, Z. G. Chen, B. Merriman, C. D. Haudenschild, S. Pradhan, et al. 2008. Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. *Nature* 452: 215–219.

Cortijo, S., R. Wardenaar, M. Colomé-Tatché, A. Gilly, M. Etcheverry, K. Labadie, E. Caillieux, et al. 2014. Mapping the epigenetic basis of complex traits. *Science* 343: 1145–1148.

Davies, S. W., S. V. Scarpino, T. Pongwarin, J. Scott, and M. V. Matz. 2015. Estimating trait heritability in highly fecund species. *G3 (Bethesda)* 5: 2639–2645.

Everitt, B. S. 2006. The Cambridge dictionary of statistics. Cambridge University Press, Cambridge, United Kingdom.

Foust, C. M., V. Preite, A. W. Schrey, M. Alvarez, M. H. Robertson, K. J. V. Verhoeven, and C. L. Richards. 2016. Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. *Molecular Ecology* 25: 1639–1652.

Fulneček, J., and A. Kovařik. 2014. How to interpret Methylation Sensitive Amplified Polymorphism (MSAP) profiles? *BMC Genetics* 15: 2.

Furrow, R. E. 2014. Epigenetic inheritance, epimutation, and the response to selection. *PLoS One* 9: e101559.

Gehring, M., and S. Henikoff. 2007. DNA methylation dynamics in plant genomes. *Biochimica et Biophysica Acta* 1769: 276–286.

Geoghegan, J. L., and H. G. Spencer. 2013. Exploring epiallele stability in a population-epigenetic model. *Theoretical Population Biology* 83: 136–144.

Gorelick, R. 2005. Environmentally alterable additive genetic effects. *Evolutionary Ecology Research* 7: 371–379.

Gorelick, R., and M. D. Laubichler. 2008. Genetic = Heritable (Genetic ≠ DNA). *Biological Theory* 3: 79–84.

Grant-Downton, R. T., and H. G. Dickinson. 2005. Epigenetics and its implications for plant biology. 1. The epigenetic network in plants. *Annals of Botany* 96: 1143–1164.

Grant-Downton, R. T., and H. G. Dickinson. 2006. Epigenetics and its implications for plant biology 2. The 'epigenetic epiphany': Epigenetics, evolution and beyond. *Annals of Botany* 97: 11–27.

Grativol, C., A. S. Hemerly, and P. C. Gomes Ferreira. 2012. Genetic and epigenetic regulation of stress responses in natural plant populations. *Biochimica et Biophysica Acta* 1819: 176–185.

Guevara, M. A., de María N., E. Sáez-Laguna, M. D. Vélez, M. T. Cervera, and J. A. Cabezas. 2017. Analysis of DNA cytosine methylation patterns using methylation-sensitive amplification polymorphism (MSAP). *In* I. Kovalchuk [ed.], Plant epigenetics. Methods and protocols, 99–112. Humana Press, New York, New York.

Hauser, M. T., W. Aufsatz, C. Jonak, and C. Luschnig. 2011. Transgenerational epigenetic inheritance in plants. *Biochimica et Biophysica Acta* 1809: 459–468.

Herrera, C. M., and P. Bazaga. 2016. Genetic and epigenetic divergence between disturbed and undisturbed subpopulations of a Mediterranean shrub: a 20-year field experiment. *Ecology and Evolution* 6: 3832–3847.

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

Herrera, C. M., M. Medrano, and P. Bazaga. 2014. Variation in DNA methylation transmissibility, genetic heterogeneity and fecundity-related traits in natural populations of the perennial herb *Helleborus foetidus*. *Molecular Ecology* 23: 1085–1095.

Herrera, C. M., M. Medrano, and P. Bazaga. 2016. Comparative spatial genetics and epigenetics of plant populations: heuristic value and a proof of concept. *Molecular Ecology* 25: 1653–1664.

Herrera, C. M., M. Medrano, and P. Bazaga. 2017. Epigenetic and genetic spatial structure of the perennial herb *Helleborus foetidus*: isolation by environment, isolation by distance, and functional trait divergence. *American Journal of Botany* 104: 1195–1204.

Hu, Y. D., G. Morota, G. J. M. Rosa, and D. Gianola. 2015. Prediction of plant height in *Arabidopsis thaliana* using DNA methylation data. *Genetics* 201: 779–793.

Jablonka, E., and M. J. Lamb. 2015. The inheritance of acquired epigenetic variations. *International Journal of Epidemiology* 44: 1094–1103.

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

Kawashima, T., and F. Berger. 2014. Epigenetic reprogramming in plant sexual reproduction. *Nature Reviews Genetics* 15: 613–624.

Kondo, H., H. Ozaki, K. Itoh, A. Kato, and K. Takeno. 2006. Flowering induced by 5-azacytidine, a DNA demethylating reagent in a short-day plant, *Perilla frutescens* var. *crispa*. *Physiologia Plantarum* 127: 130–137.

Kooke, R., F. Johannes, R. Wardenaar, F. Becker, M. Etcheverry, V. Colot, D. Vreugdenhil, and J. J. B. Keurentjes. 2015. Epigenetic basis of morphological variation and phenotypic plasticity in *Arabidopsis thaliana*. *Plant Cell* 27: 337–348.

Kronholm, I., and S. Collins. 2016. Epigenetic mutations can both help and hinder adaptive evolution. *Molecular Ecology* 25: 1856–1868.

Lauria, M., S. Piccinini, R. Pirona, G. Lund, A. Viotti, and M. Motto. 2014. Epigenetic variation, inheritance, and parent-of-origin effects of cytosine methylation in maize (*Zea mays*). *Genetics* 196: 653–666.

Lerceteau, E., and A. E. Szmidt. 1999. Properties of AFLP markers in inheritance and genetic diversity studies of *Pinus sylvestris* L. *Heredity* 82: 252–260.

Lister, R., R. C. O'Malley, J. Tonti-Filippini, B. D. Gregory, C. C. Berry, A. H. Millar, and J. R. Ecker. 2008. Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell* 133: 523–536.

Lukens, L. N., and S. H. Zhan. 2007. The plant genome's methylation status and response to stress: implications for plant improvement. *Current Opinion in Plant Biology* 10: 317–322.

Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer, Sunderland, Massachusetts, USA.

Maughan, P. J., M. A. Saghai Maroof, G. R. Buss, and G. M. Huestis. 1996. Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. *Theoretical and Applied Genetics* 93: 392–401.

Medrano, M., C. M. Herrera, and P. Bazaga. 2014. Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. *Molecular Ecology* 23: 4926–4938.

Messeguer, R., M. W. Ganal, J. C. Steffens, and S. D. Tanksley. 1991. Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. *Plant Molecular Biology* 16: 753–770.

Miska, E. A., and A. C. Ferguson-Smith. 2016. Transgenerational inheritance: models and mechanisms of non-DNA sequence-based inheritance. *Science* 354: 59–63.

Osabe, K., J. D. Clement, F. Bedon, F. A. Pettolino, L. Ziolkowski, D. J. Llewellyn, E. J. Finnegan, and I. W. Wilson. 2014. Genetic and DNA methylation changes in cotton (*Gossypium*) genotypes and tissues. *PLoS One* 9: e86049.

Pecinka, A., and O. Mittelsten Scheid. 2012. Stress-induced chromatin changes: a critical view on their heritability. *Plant and Cell Physiology* 53: 801–808.

Quadrana, L., and V. Colot. 2016. Plant transgenerational epigenetics. *Annual Review of Genetics* 50: 467–491.

R Development Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at https://www.R-project.org/.

Rensing, S. A. 2017. Why we need more non-seed plant models. *New Phytologist* 216: 355–360.

Revelle, W. 2017. psych: Procedures for personality and psychological research, Northwestern University, Evanston, Illinois. Available at https://CRAN.R-project.org/package=psych

Richards, E. J. 1997. DNA methylation and plant development. *Trends in Genetics* 13: 319–323.

Roff, D. A. 1997. Evolutionary quantitative genetics. Chapman and Hall, New York, New York.

Rousseeuw, P. J., and A. M. Leroy. 1987. Robust regression and outlier detection. Wiley, New York.

Rozhon, W., T. Baubec, J. Mayerhofer, O. Mittelsten Scheid, and C. Jonak. 2008. Rapid quantification of global DNA methylation by isocratic cation exchange high-performance liquid chromatography. *Analytical Biochemistry* 375: 354–360.

Sano, H., I. Kamada, S. Youssefian, M. Katsumi, and H. Wabiko. 1990. A single treatment of rice seedlings with 5-azacytidine induces heritable dwarfism and undermethylation of genomic DNA. *Molecular and General Genetics* 220: 441–447.

Schmitz, R. J., M. D. Schultz, M. G. Lewsey, R. C. O'Malley, M. A. Urich, O. Libiger, N. J. Schork, and J. R. Ecker. 2011. Transgenerational epigenetic instability is a source of novel methylation variants. *Science* 334: 369–373.

Schrey, A. W., M. Alvarez, C. M. Foust, H. J. Kilvitis, J. D. Lee, A. L. Liebl, L. B. Martin, et al. 2013. Ecological epigenetics: beyond MS-AFLP. *Integrative and Comparative Biology* 53: 340–350.

Schulz, B., R. L. Eckstein, and W. Durka. 2013. Scoring and analysis of methylation-sensitive amplification polymorphisms for epigenetic population studies. *Molecular Ecology Resources* 13: 642–653.

Schulz, B., R. L. Eckstein, and W. Durka. 2014. Epigenetic variation reflects dynamic habitat conditions in a rare floodplain herb. *Molecular Ecology* 23: 3523–3537.

Shea, N., I. Pen, and T. Uller. 2011. Three epigenetic information channels and their different roles in evolution. *Journal of Evolutionary Biology* 24: 1178–1187.

Steward, N., M. Ito, Y. Yamaguchi, N. Koizumi, and H. Sano. 2002. Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. *Journal of Biological Chemistry* 277: 37741–37746.

Tatra, G. S., J. Miranda, C. C. Chinnappa, and D. M. Reid. 2000. Effect of light quality and 5-azacytidine on genomic methylation and stem elongation in two ecotypes of *Stellaria longipes*. *Physiologia Plantarum* 109: 313–321.

Uller, T., S. English, and I. Pen. 2015. When is incomplete epigenetic resetting in germ cells favoured by natural selection? *Proceedings of the Royal Society B* 282: 20150682.

van der Graaf, A., R. Wardenaar, D. A. Neumann, A. Taudt, R. G. Shaw, R. C. Jansen, R. J. Schmitz, et al. 2015. Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. *Proceedings of the National Academy of Sciences USA* 112: 6676–6681.

Vazquez, A. I., D. Gianola, D. Bates, K. A. Weigel, and B. Heringstad. 2009. Assessment of Poisson, logit, and linear models for genetic analysis of clinical mastitis in Norwegian Red cows. *Journal of Dairy Science* 92: 739–748.

Vekemans, X., T. Beauwens, M. Lemaire, and I. Roldán-Ruiz. 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, K. J. F., J. J. Jansen, P. J. van Dijk, and A. Biere. 2010. Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytologist* 185: 1108–1118.

Vidalis, A., D. Živković, R. Wardenaar, D. Roquis, A. Tellier, and F. Johannes. 2016. Methylome evolution in plants. *Genome Biology* 17: 264.

Villemereuil, P., O. Gimenez, and B. Doligez. 2013. Comparing parent-offspring regression with frequentist and Bayesian animal models to estimate heritability in wild populations: a simulation study for Gaussian and binary traits. *Methods in Ecology and Evolution* 4: 260–275.

Wilschut, R. A., C. Oplaat, L. B. Snoek, J. Kirschner, and K. J. F. Verhoeven. 2016. Natural epigenetic variation contributes to heritable flowering divergence in a widespread asexual dandelion lineage. *Molecular Ecology* 25: 1759–1768.

Zemach, A., I. E. McDaniel, P. Silva, and D. Zilberman. 2010. Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science* 328: 916–919.

Zheng, X., L. Chen, H. Xia, H. Wei, Q. Lou, M. Li, T. Li, and L. Luo. 2017. Transgenerational epimutations induced by multi-generation drought imposition mediate rice plant's adaptation to drought condition. *Scientific Reports* 7: 39843.