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Within-plant variation in seed size and inflorescence fecundity is associated with epigenetic mosaicism in the shrub *Lavandula latifolia* (Lamiaceae)

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• **Background and Aims** Sub-individual variation in traits of homologous structures has multiple ecological consequences for individuals and populations. Assessing the evolutionary significance of such effects requires an improved knowledge of the mechanisms underlying within-plant phenotypic heterogeneity. The hypothesis that continuous within-plant variation in some phenotypic traits can be associated with epigenetic mosaicism was examined.
• **Methods** Fifteen individuals of the long-lived, evergreen Mediterranean shrub *Lavandula latifolia* were studied. Five widely spaced 'modules', each consisting of a single inflorescence plus all its subtending basal leaves, were collected from each shrub. Genomic DNA was extracted from leaf samples and genome-wide cytosine methylation determined by reversed phase high-performance liquid chromatography (HPLC) with spectrofluorimetric detection. The number and mean mass of seeds produced were determined for each inflorescence. An assessment was made of whether (1) leaves from different modules in the same plant differed significantly in global DNA cytosine methylation, and (2) mosaicism in cytosine methylation contributed to explain variation across modules in number and size of seeds.
• **Key Results** Leaves from different modules in the same plant differed in global DNA cytosine methylation. The magnitude of epigenetic mosaicism was substantial, as the variance in DNA methylation among modules of the same shrub was greater than the variance between individuals. Number and mean mass of seeds produced by individual inflorescences varied within plants and were quadratically related to cytosine methylation of subtending leaves, with an optimum at an intermediate methylation level (approx. 25 %).
• **Conclusions** The results support a causal link between global cytosine methylation of leaves in a module and the size and numbers of seeds produced by the associated inflorescence. It is proposed that variation in global DNA methylation within *L. latifolia* shrubs may result from the concerted action of plant sectoriality and differential exposure of different plant parts to some environmental factor(s) with a capacity to induce durable epigenetic changes.

Key words: DNA methylation, epigenetic mosaicism, *Lavandula latifolia*, seed production, seed size, sub-individual variation

INTRODUCTION

Reiteration of homologous, functionally equivalent structures is a quintessential feature of the body plan of higher plants. Multiple homologous organs borne by an individual and performing the same function (e.g. leaves, flowers and seeds) are not phenotypically identical, which leads to a within-plant component of phenotypic variance that often contributes more to population-level variance than does variation among plants (Herrera, 2009; Herrera *et al.*, 2015). In contrast to the frequent attention received by the conspicuous, albeit infrequent, instances of discrete within-plant variation (e.g. heterophylly, heterocarpy and seed heteromorphism), the ecological and evolutionary implications of ubiquitous continuous variation within plants have traditionally received little attention, possibly because this kind of sub-individual variation was long deemed ecologically unimportant and evolutionarily irrelevant (Haldane, 1932; Stebbins, 1950). Recently, however, increasing evidence has shown that sub-individual variation in continuous traits of reiterated organs can have multiple ecological effects in plant populations (Herrera, 2009; Herrera *et al.*, 2015). These include broadening the ecological breadth of species and enhancing the functional diversity of populations and communities (Herrera *et al.*, 2015); improving the exploitation of limiting resources such as light or nitrogen (Osada *et al.*, 2014; Ponce-Bautista *et al.*, 2017); modifying the outcome of plant interactions with antagonists (Sobral *et al.*, 2014; Shimada *et al.*, 2015; Wetzel *et al.*, 2016) and mutualists (Sobral *et al.*, 2013; Benitez-Vieyra *et al.*, 2014); influencing pollinator-driven selection on phenology, mating system and floral traits (Ishii and Harder, 2012; Austen *et al.*, 2015; Screiber *et al.*, 2015; Dai *et al.*, 2016; Arceo-Gómez *et al.*, 2017); and coping with environmental uncertainty in biotic and abiotic factors (Herrera, 2009; Tíscar Oliver and Lucas Borja, 2010;

Hidalgo *et al.*, 2016). Assessing the potential evolutionary significance of these ecological effects will require an improved understanding of the mechanisms underlying within-plant phenotypic heterogeneity.

'Ontogenetic contingency' (*sensu* Diggle, 1994), which incorporates the combined effects of position in the plant or along developmental axes (architectural effects), developmental history and external environment, along with developmental stochasticity, seem the major determinants of within-plant variation in continuous phenotypic traits of reiterated structures (Herrera, 2009). Genetic mosaicism caused by somatic mutations (Whitham and Slobodchikoff, 1981) has been considered a minor source of within-plant phenotypic variation, given the extreme rarity of well-documented genetic mosaics in wild plants under natural conditions (Herrera, 2009; but see O'Connell and Ritland, 2004; Padovan *et al.*, 2013; Ranade *et al.*, 2014). Recent investigations, however, indicate that conventional genetic mosaicism is not the only possible way in which genomic heterogeneity of individual plants can bring about sub-individual phenotypic variation. Epigenetic mosaicism in which different parts of the same genetic individual differ in DNA methylation patterns has been documented a few times for clonal (Gao *et al.*, 2010; Bian *et al.*, 2013; Spens and Douhovnikoff, 2016) and non-clonal plants (Bitonti *et al.*, 1996; Herrera and Bazaga, 2013). For example, in the heterophyllous tree *Ilex aquifolium*, pairs of contiguous prickly and non-prickly leaves along the same branchlet differed in genome-wide patterns of DNA methylation (Herrera and Bazaga, 2013). Given also the frequent causal association between DNA methylation variants and differences between individuals in continuous phenotypic traits (Johannes *et al.*, 2009; Zhang *et al.*, 2013; Cortijo *et al.*, 2014; Hu *et al.*, 2015; Kooke *et al.*, 2015), we hypothesized that epigenetic mosaicism caused by sub-individual heterogeneity in DNA methylation levels may be associated with within-plant variation in some continuous phenotypic traits.

Expectations from the preceding hypothesis are examined herein by (1) assessing whether leaves borne by different parts of the crown of the long-lived, evergreen Mediterranean shrub *Lavandula latifolia* differed significantly in global DNA cytosine methylation (= proportion of all genomic cytosines that are methylated); and (2) testing whether mosaicism in the genome-wide cytosine methylation level contributes to explain the broad sub-individual variation in size and number of seeds produced by individual inflorescences which are characteristic of this species (Herrera 1991, 2000). Cytosine methylation is key for epigenetic regulation in plants (Finnegan *et al.*, 1998; Grant-Downton and Dickinson, 2005, 2006), playing important roles in gene expression, genomic integrity, and plant growth and development (Richards, 1997; Finnegan *et al.*, 2000; Cokus *et al.*, 2008; Lister *et al.*, 2008). Intraspecific variation in global cytosine methylation is usually associated with changes in the methylation status of specific genic and intergenic regions, and has functional consequences in terms of altered gene expression and genomic instability (Steward *et al.*, 2002; Baubec *et al.*, 2009; Bonchev and Parisod, 2013; Vidalis *et al.*, 2016). These observations motivate our expectation that epigenetic mosaicism could produce small-scale, within-plant phenotypic mosaicism in plants.

MATERIALS AND METHODS

Lavandula latifolia Med. is a dome-shaped, long-lived evergreen shrub inhabiting clearings and well-lit undergrowth of mid-elevation mixed woodlands in the eastern Iberian Peninsula (Herrera and Jovani, 2010). The branching pattern is dichasial, regularly dichotomous and resembles Leeuwenberg's development model (Hallé *et al.*, 1978). Opposite axillary buds under the apical bud grow more or less symmetrically following the developmental arrest of the apical bud, eventually resulting into a fork-like division. If undisturbed, this dichotomous branching pattern eventually leads to crowns of adult plants being made up of morphological units consisting of distinct leaf clusters borne by short stems. In early summer, long-stalked inflorescences are produced at the tips of these short leafy stems (Fig. 1). During the flowering season, structural clusters thus usually consist of one inflorescence plus its associated set of subtending, even-aged leaves, which will be termed a 'module' hereafter (Fig. 1). Flowers are hermaphrodite and insect pollinated, and the species reproduces exclusively by seed. Within-plant variation in seed mass is extensive in this species, accounting for >90 % of total population-wide variance (Herrera, 2000). The number of seeds produced by each inflorescence is also highly variable among inflorescences of the same plant (>40 % of population-wide variance; Herrera, 1991).

Sampling for this study was conducted on 10–11 September 2014 at a large *L. latifolia* population growing close to Arroyo Aguaderillos in the Sierra de Cazorla (Jaén province, south-eastern Spain). Fifteen widely spaced shrubs (range of pairwise distances = 10–180 m) roughly similar in size were selected for study. Five modules, each consisting of a single inflorescence plus all its subtending basal leaves (Fig. 1), were collected from each shrub. In each plant, sampled modules represented <15 % of all inflorescence-bearing modules (typically 25–50; Herrera and Jovani, 2010) and were distributed as evenly as possible across the shrub's crown to minimize the effects of possible spatial autocorrelation within plants. Leaves were placed in paper envelopes and dried at ambient temperature in containers with silica gel for subsequent DNA extraction. When collections were conducted, the flowering season was nearly finished and inflorescences were soon to start shedding ripe seeds. Collected inflorescences were placed individually inside fine mesh bags, and kept indoors at room temperature for several weeks until all ripe seeds had been liberated naturally. Ripe seeds from each collected inflorescence were then counted and weighed collectively, and the mean seed mass for each module was computed from these figures.

Genome-wide percentage methylation of DNA cytosines was determined separately for the leaves of each module using the chromatographic technique described in detail by Alonso *et al.* (2016). Total genomic DNA was extracted from dried leaf samples using the Bioline ISOLATE II Plant DNA Kit, and digested with DNA Degradase Plus™ (Zymo Research, Irvine, CA, USA), a nuclease mix that degrades DNA to its individual nucleoside components. Digested samples were stored at –20 °C until analysed. To allow for testing the statistical significance of within-plant heterogeneity (i.e. between modules) in DNA methylation level, two independent technical replicates of DNA hydrolysate were prepared for each module, and the 150 samples (15 plants × 5 modules × 2 replicates) were processed in randomized order. DNA cytosine methylation was determined for each sample by

FIG. 1. Flowering *Lavandula latifolia* shrub (left) and idealized drawing of one of its modules, consisting of a single inflorescence plus its subtending leaves (right).

reversed phase high-performance liquid chromatography (HPLC) with spectrofluorimetric detection. Global cytosine methylation was estimated for each sample 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).

Statistical significance tests of heterogeneity in global cytosine methylation among individual plants, and among different modules of the same plant, were conducted by fitting a linear model to the data, treating plants and modules nested within plants as fixed-effect predictors. The contributions of differences between plants, and between modules within the same plant, to total sample variance in per module mean seed mass and seed number, and global cytosine methylation, were estimated by fitting an intercept-only random effect model to the data, with plants and modules as hierarchically nested random effects. Relationships between global cytosine methylation and fecundity-related traits were tested by fitting two linear mixed-effects models to the data, treating modules as the sampling units. The total number of seeds produced per module (log transformed) and mean seed mass per module were the response variables in these models. Cytosine methylation per module (mean value of the two independent replicates) and its square (to account for non-linearity) were the predictors, and plants were included as a random effect. Random intercepts models were fitted in all instances, since more complex random intercepts and slopes models did not significantly improve fit in any case, as tested with ordinary likelihood ratio tests. It must be stressed that, since plants were treated as random effects, these mixed models tested associations between response variables and global cytosine methylation across modules after statistically controlling for differences in plant means. The relationships linking per module fecundity traits and global cytosine methylation were visualized by plotting model-predicted values of response variables against global cytosine methylation for the whole sample (fixed effects) and stratified by individual plants (random effect). All statistical analyses were carried out using the R environment (R Development Core Team, 2016). The lmer function from the lme4 package was used to fit random and mixed-effect models (Bates *et al.*, 2015). Function sjp.lmer from the sjPlot package (Lüdecke, 2017) was used to check assumptions of mixed-effects models (Pinheiro and Bates, 2000).

RESULTS

In most plants sampled, both the number and the mean mass of seeds produced by single inflorescences varied widely among modules of the same individual (Supplementary Data Table S1; Fig. S1). Total sample variance in per module mean seed mass and seed production was mostly accounted for by differences between modules of the same shrub (75.8 and 54.8 % of total sample variance, respectively).

Global cytosine methylation of leaf genomes varied among plants and among modules of the same plant (Fig. 2), differences being highly significant at the two hierarchical levels considered ($F = 7.49$, d.f. = 14, 74, $P < 0.0001$; and $F = 3.76$, d.f. = 60, 74, $P < 0.0001$; between plants, and between modules within plants, respectively). Partitioning of total variance in global cytosine methylation occurring in our sample into between- and within-plant components revealed that it was primarily accounted for by variation between modules of the same plant (variance = 1.748, or 50.28 % of total), and only secondarily by variation between plant means (variance = 0.471, or 13.54 % of total). This finding reveals a substantial role for sub-individual variation as a source of population-level variance in global DNA methylation in the set of *L. latifolia* plants sampled.

The number and mean mass of seeds produced by individual modules were significantly related to the modules' global

cytosine methylation, as revealed by results of linear mixed-effects models. After statistically accounting for individual differences by including plants as a random effect in models, there were highly significant quadratic relationships between global cytosine methylation of genomic DNA from a module's leaves and both the total number of seeds (log transformed) and the mean mass of individual seeds produced by the associated inflorescence (Table 1). Model-fitted equations for the whole sample and predicted responses for individual plants (Fig. 3) revealed that the number and mean size of seeds produced by individual modules were greatest at intermediate cytosine methylation levels, and declined as the methylation level increased or decreased slightly from that optimum value, which occurred at around 25 %.

The quadratic relationship across modules linking mean seed mass and mean cytosine methylation also held across plant mean values, with individuals exhibiting intermediate mean methylation levels (approx. 25 %) producing the heaviest seeds (Fig. 4). It was not possible to examine the relationship across plants between total seed production and cytosine methylation level, as estimates of total seed production per plant were not available.

DISCUSSION

Global cytosine methylation measurements do not provide information on the genomic positions at which methylation occurs, but its analysis represents an initial step to evaluate the

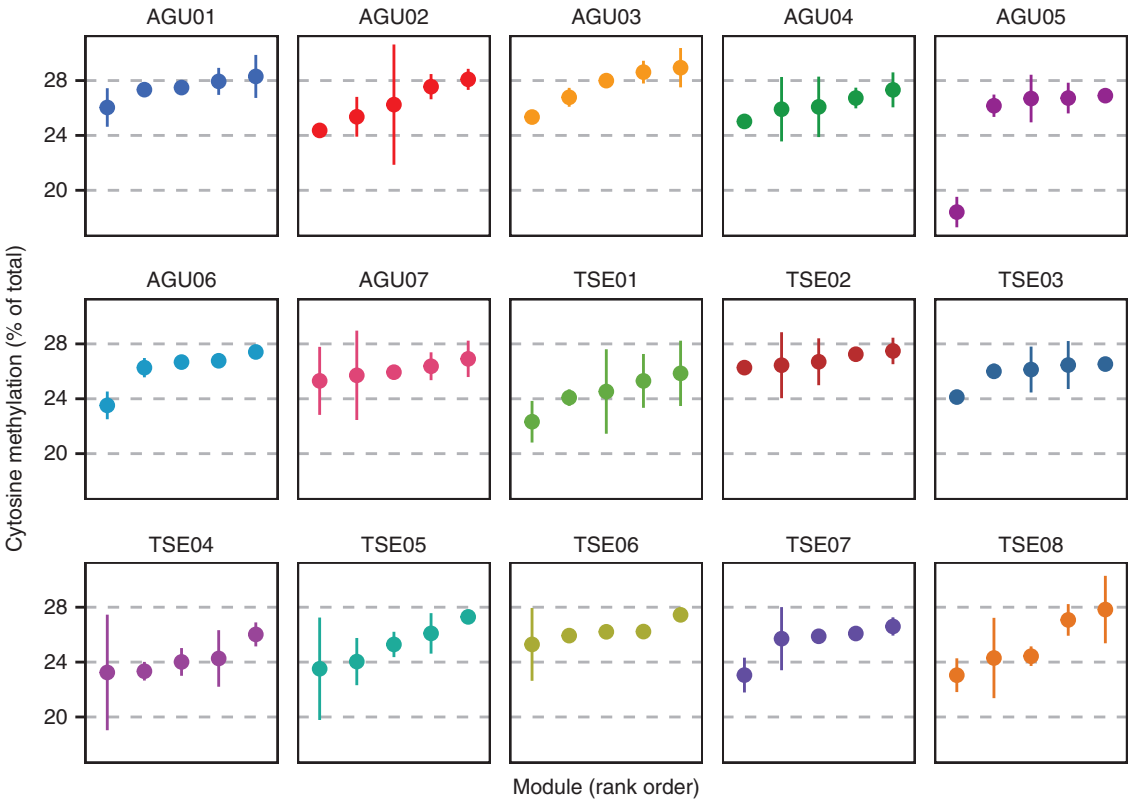

FIG. 2. Variation in global DNA cytosine methylation across *Lavandula latifolia* plants (AGU01–TSE08) and between different modules from the same plant (five modules sampled per plant). Within each panel, modules were ranked in increasing order of mean cytosine methylation. Dots represent average values of the two independent replicates run for each module, and vertical segments extend ± 2 s.e. around the mean.

TABLE 1. *Summary of results of linear mixed-effects models testing for linear and quadratic effects of global cytosine methylation on the number and mean mass of seeds produced by individual modules of* Lavandula latifolia *plants*

	Predictor					
	Global cytosine methylation			(Global cytosine methylation)^2		
Response variable	Standardized coefficient ± s.e.	χ^2	*P*-value	Standardized coefficient ± s.e.	χ^2	*P*-value
Log10 (no. of seeds)	+4.282 ± 1.384	9.57	0.0020	–4.068 ± 1.397	8.47	0.0036
Mean seed mass	+4.873 ± 1.587	9.43	0.0021	–4.965 ± 1.597	9.67	0.0019

Plants were included in the models as a random effect.

See Fig. 3 for the shape of relationships.

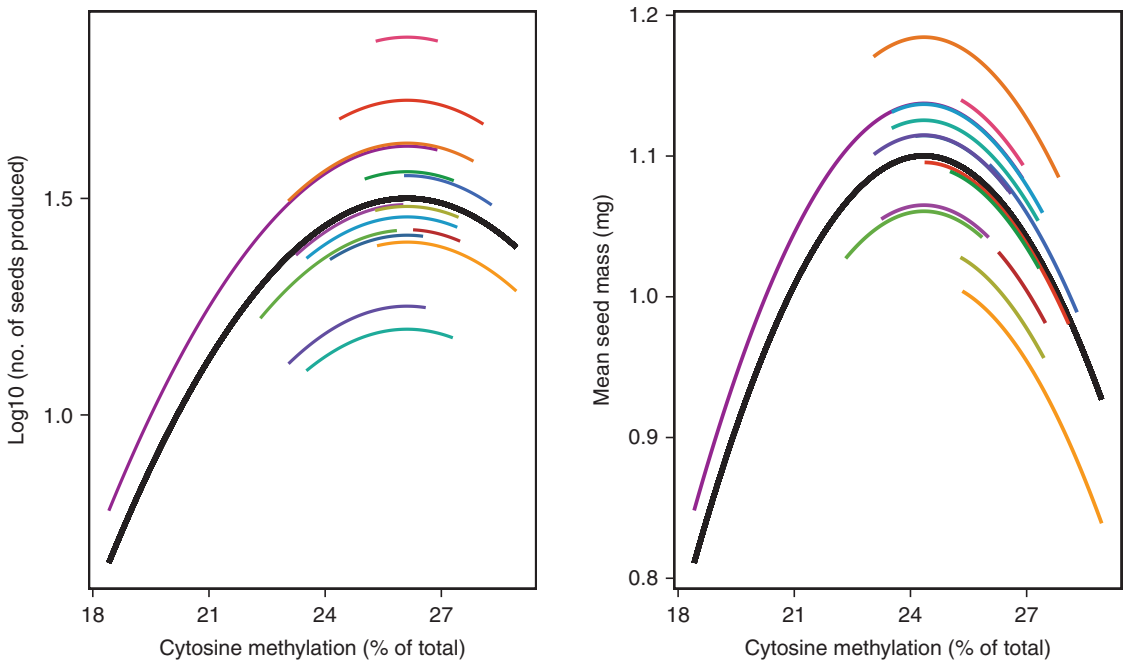

FIG. 3. Model-fitted quadratic equations depicting the relationships between the number (log transformed) and mean mass of seeds produced by individual modules, and global cytosine methylation of genomic DNA from the modules' leaves. Predicted relationships are shown for the sample as a whole (i.e. the fixed effects part in the mixed-effects model, thick black lines) and for each plant separately (i.e. the prediction for each random effect level, thin lines; plant colour codes as in Fig. 2).

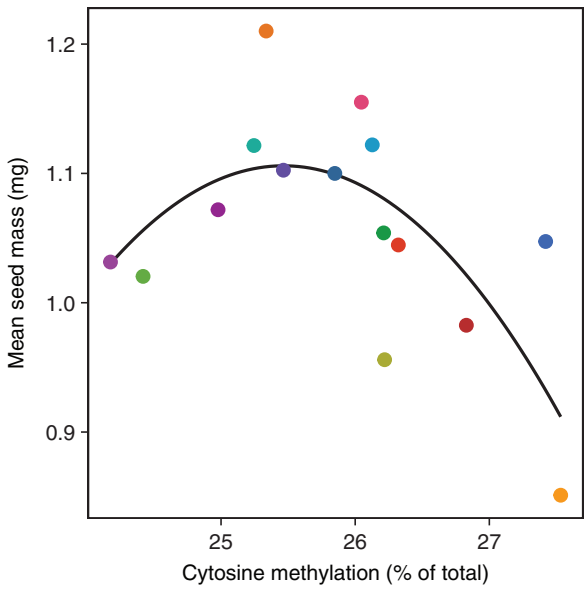

FIG. 4. Quadratic relationship between mean seed mass and mean global DNA cytosine methylation across the 15 *Lavandula latifolia* plants sampled ($y = -28.392 + 2.317x - 0.045x^2$; $F = 5.79$, d.f. = 2, 12, $P = 0.017$, adjusted $R^2 = 0.406$). Plant means were obtained by averaging across modules within each plant. Colour codes for plants as in Fig. 2.

possible role of this epigenetic mark for non-model organisms when detailed genomic information is missing (Rozhon *et al.*, 2008; Alonso *et al.*, 2016). Intraspecific variation in global cytosine methylation is associated with changes in the methylation status of specific genic and intergenic regions, and has functional consequences in terms of altered gene expression and genomic instability (see references in the Introduction), as well as effects on phenotypic traits such as plant size, fecundity or time to flowering (Sano *et al.*, 1990; Tatra *et al.*, 2000; Kondo *et al.*, 2006). Individual differences in levels of genome-wide cytosine methylation have been found associated with variation in fecundity-related traits in natural populations of the perennial herb *Helleborus foetidus* (Alonso *et al.*, 2014). The present study confirms and expands these results for a long-lived shrub by demonstrating associations between genome-wide methylation and seed size and numbers, across individuals and across modules of the same individual.

Our results confirmed the hypothesis of sub-individual epigenetic mosaicism for *L. latifolia*. Samples of even-aged leaves from distinct modules in the same plant differed significantly in global DNA cytosine methylation. The magnitude of within-plant variation was substantial, as variance in DNA methylation among modules of the same individual exceeded the variance among individuals. Apart from their biological interest, discussed below, these results prompt some methodological considerations for future epigenetic studies. Given the quantitative importance of within-plant variance in the DNA methylation level found here for *L. latifolia*, adequate epigenetic characterization of individual plants may require repeated within-plant sampling following a well-planned stratified design. This is expected to apply particularly to large-sized, long-lived shrubs and trees, as these are possibly most susceptible to the appearance of epigenetic mosaicism in response to spatio-temporal heterogeneity of the environment experienced over their lifetimes (see below). Pooled DNA samples from leaves (or other organs) collected from different parts of the same individual can be heterogeneous with regard to the genome-wide cytosine

methylation level, as shown by this study. When subjected to analytical procedures based on the application of restriction enzymes (e.g. the methylation-sensitive amplified fragment polymorphisms method, Schulz *et al.*, 2013; reduced representation bisulphite sequencing, Gu *et al.*, 2011), heterogeneous DNA samples consisting of a mixture of epigenotypes will be prone to impaired amplification and poor repeatability. Collecting and analysing DNA samples for different plant parts separately, particularly in the case of large long-lived plants as noted above, may help to alleviate technical problems derived from DNA heterogeneity in methylation status.

Within species, global DNA methylation may vary with plant age and tissue of origin (Fraga *et al.*, 2002; Mankessi *et al.*, 2011; Vining *et al.*, 2012). These factors, however, cannot account for the extensive variation in genomic methylation between modules of the same plant found in this study, since all DNA samples were obtained at the same time from an even-aged leaf cohort. We propose rather that sub-individual variation in global DNA methylation in *L. latifolia* is a consequence of the concerted action of plant sectoriality and long-term differential exposure of distinct plant parts to one or more environmental factors with a capacity to induce persistent changes in global cytosine methylation (for a review, see Alonso *et al.*, 2016). The latter include pathogens, herbivores, insolation, UV light, water shortage and nitrogen deficiency, all of which are known to induce stable epigenetic responses in plants (Boyko *et al.*, 2007; Herrera and Bazaga, 2011; Kou *et al.*, 2011; Ohlsson *et al.*, 2013; Kinoshita and Seki, 2014; Muller-Xing *et al.*, 2014).

Central to the preceding hypothesis is plant sectoriality, the phenomenon whereby plant bodies are compartmentalized into relatively independent physiological sub-units that may behave semi-autonomously ('integrated physiological units'; Watson, 1986). Distinct aerial sectors of a plant are connected to distinct sectors of the root system that may differ in their acquisition of water or nutrients (Orians *et al.*, 2002). Also, herbivores and pathogens may in the long run favour certain parts of individuals over others, and different plant parts are consistently exposed to contrasting insolation, photosynthesis and transpiration regimes (e.g. north vs. south exposure). Altogether, different parts of the same individual will in the long term experience chronically different levels of environmental stress and may develop small-scale differences in genomic methylation as a consequence of localized epigenetic responses. By constraining the horizontal circulation of the set of phloem-mobile molecules that regulate genome-wide DNA methylation (Molnar *et al.*, 2010; McGarry and Kragler, 2013; Lewsey *et al.*, 2016), sectoriality of phloem transport will contribute to perpetuate small-scale, within-plant variation in genomic methylation arising from localized responses to environmental agents, as previously suggested in relation to other sub-individually variable traits (Orians and Jones, 2001; Herrera, 2009). Under this hypothesis, the testable prediction can be formulated that interspecific differences in degree of sectoriality (Zanne *et al.*, 2006) should be related to variations in the extent of epigenetic mosaicism.

The total number and mean mass of seeds produced by individual modules, two functional traits with immediate consequences for plant reproductive success (Pérez-Harguindeguy *et al.*, 2013), varied among modules of the same plant. Such sub-individual variation was significantly associated with differences between modules in global cytosine methylation. It is not possible with the data available to establish whether these results actually reflect a causal link between global cytosine methylation of leaves in a module and seed fecundity of its associated inflorescence, since the observed association could reflect co-ordinated responses to some unmeasured factor. Demonstration of a causative connection between the DNA methylation level of leaves in a module and features of their seeds will require experimentation involving controlled manipulation of DNA methylation and testing for its putative effects on seeds. Keeping this important caveat in mind, however, some circumstantial evidence is compatible with a possible causal link between global cytosine methylation of the leaves in a module and the size and numbers of seeds produced by its associated inflorescence. In *Brassica rapa*, experimental application of a demethylating agent results in decreased seed size and seed set (Amoah *et al.*, 2012). In *Arabidopsis thaliana*, DNA methylation regulates maternal control of seed size (FitzGerald *et al.*, 2008), and hypomethylation of the maternal genome results in larger and heavier seeds, while hypomethylation of the paternal genome results in smaller, lighter seeds (Xiao *et al.*, 2006). Both effects could be involved in the observed relationship between methylation and seed size across *L. latifolia* modules, since the species is self-compatible and an unknown fraction of seeds surely results from self-fertilization. If a causal link between global cytosine methylation of individual modules and seed number and size were eventually proven, then epigenetic mosaicism would emerge as a powerful, hitherto unrecognized factor contributing to the functional diversity of plant populations through enhancement of the sub-individual component of phenotypic variation (Herrera and Bazaga, 2013; Herrera *et al.*, 2015).

Concluding remarks

Shrubs of *L. latifolia* were internally heterogenous with regard to the level of genome-wide cytosine methylation. Such sub-individual genomic heterogeneity was non-linearly related to within-plant heterogeneity in seed size and per inflorescence fecundity. Regardless of whether observed associations across modules between DNA methylation and seed size and numbers reflect causality, the finding that intermediate methylation levels were at a relative fecundity advantage was an important result of this study. Statistically significant quadratic relationships held at both between-module and between-individual levels, and small shifts towards under- or overmethylation relative to approx. 25 % were predicted to result in impaired seed size and reduced seed number at both levels. Although the underlying molecular mechanisms leading to these intriguing results cannot be established at present, they provide the first evidence to date of fecundity-mediated, stabilizing phenotypic selection acting on the global DNA methylation level in a natural plant population and support the view that it is an evolving trait in angiosperms (Alonso *et al.*, 2015). Stabilizing selection on DNA methylation was particularly strong in *L. latifolia*, as denoted by the absolute values of standardized quadratic regression coefficients exceeding those commonly reported by phenotypic selection studies on plant morphology, phenology and life history traits (see, for example, Kingsolver and Diamond, 2011).

The significance of this finding will ultimately depend on the transmission of global DNA methylation from maternal parents to offspring, an aspect unknown so far for *L. latifolia* or any other non-model plant. Experiments are currently underway to assess the transgenerational inheritance of global cytosine methylation in *L. latifolia* and evaluate whether the epigenetic mosaicism documented here does eventually translate into epigenetically heterogeneous progeny.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: raw data used in the study, consisting of mean global methylation, seed number and mean seed mass, per individual module. Figure S1: variation in mean seed mass and total number of seeds across *Lavandula latifolia* plants and between modules of the same plant.

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