

Evidence for an epigenetic role in inbreeding depression



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Inbreeding depression (i.e. negative fitness effects of inbreeding) is central in evolutionary biology, affecting numerous aspects of population dynamics and demography, such as the evolution of mating systems, dispersal behaviour and the genetics of quantitative traits. Inbreeding depression is commonly observed in animals and plants. Here, we demonstrate that, in addition to genetic processes, epigenetic processes may play an important role in causing inbreeding effects. We compared epigenetic markers of outbred and inbred offspring of the perennial plant *Scabiosa columbaria* and found that inbreeding increases DNA methylation. Moreover, we found that inbreeding depression disappears when epigenetic variation is modified by treatment with a demethylation agent, linking inbreeding depression firmly to epigenetic variation. Our results suggest an as yet unknown mechanism for inbreeding effects and demonstrate the importance of evaluating the role of epigenetic processes in inbreeding depression.

Keywords: DNA methylation; epigenetics; inbreeding depression; *Scabiosa columbaria*; 5-azacytidine

1. INTRODUCTION

Negative effects of inbreeding have until now been exclusively explained by classic genetic theories: the partial dominance hypothesis, i.e. the expression of deleterious recessive alleles owing to increased homozygosity in inbred individuals [1]; and the overdominance hypothesis, i.e. the reduced frequency of superior heterozygote genotypes [1]. In addition, interacting effects between alleles at different loci (i.e. epistasis) have been suggested to contribute to inbreeding depression [2], as is interpreted from nonlinear relationships between inbreeding level and fitness [3]. There is consensus that partial dominance is the most likely explanation in most cases.

Inbreeding effects are often found to be environmentally dependent [4–8]. Environment-dependent inbreeding depression can be explained by different mechanisms [5,7], including the conditional expression of deleterious alleles, and the different levels of phenotypic plasticity expressed by outbred and inbred individuals [9]. We know that epigenetic modifications, chemical modifications to the DNA or histones that alter or regulate gene activity, are affected by environmental conditions and may modulate plasticity [10].

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Some of these environmentally induced modifications are heritable over multiple generations [11] and variation in these modifications among individuals and populations [12,13] has been related to differences in phenotype [14], development and even mortality [15]. It has therefore been suggested that epigenetic processes are involved in the regulation of inbreeding effects [16,17]. However, empirical proof for this is, as yet, not available.

To investigate the link between inbreeding, epigenetic processes and inbreeding depression, we studied the perennial plant *Scabiosa columbaria*, a species known to suffer severely from inbreeding depression [18]. We tested whether inbred and outbred plants differ in levels of DNA methylation and whether modifying DNA methylation of inbred and outbred plants affects the phenotypic differences observed between inbred and outbred individuals.

2. METHODS

Scabiosa columbaria is a self-compatible but predominantly outcrossing species with outcrossing rates close to 1 in natural populations [19]. In 2009, seeds were collected from a large French population (more than 100 000 individuals). These seeds were germinated and grown until flowering. The individual plants were both selfed and outcrossed by pollinating at least four flower heads per plant to create inbred and outbred siblings. From the F₁ generation, 75 plants (38 outbred and 37 inbred siblings from six families, *ca* six to eight replicates per family with inbreeding coefficients of 0 and 0.5, respectively) were grown until flowering on a 1:3 compost:sand mixture, day/night temperatures of 24°C/18°C, 16 h photoperiod and 60 per cent relative humidity. To investigate the effect of inbreeding on DNA methylation, a key epigenetic mechanism [20], we experimentally demethylated half of our plants (*ca* three to four replicates per family) by germinating seeds on filter paper saturated with a daily-refreshed 50 µM 5-azacytidine [21] solution, applied for 9 days. The demethylating effect of 5-azacytidine has been demonstrated by others [21–23].

After three months, plant biomass was estimated non-destructively as the product of leaf number and the length and width of the largest leaf, which was highly correlated with actual biomass in a separate set of plants (Pearson correlation, $R^2 = 0.79$, $n = 96$, $p < 0.001$). Photosynthesis light responses from 1500 to 0 photosynthetically active radiation (PAR) were measured between 10.00 and 15.00 on two and a half month-old plants using a LiCor LI-6400 (Lincoln, NB, USA), at 20°C, 60 per cent relative humidity, and 400 µmol mol⁻¹ CO₂ as reference. Photosynthetic efficiency was determined by fitting the slope between 0 and 60 PAR. Inbreeding depression coefficients were calculated as $\delta = (W_O - W_S)/W_O$, with W_O and W_S the mean values of a particular trait of outcrossed and selfed offspring, respectively. Inbreeding depression coefficients for bolting time were multiplied by -1 because high trait values indicate poor performance.

Mixed effect models (R-2.14.1; lme-package) with family included as random effect were used to test for effects of crossing type, demethylation and their interaction on the response variables. Model validation gave no indication of nonlinearity. Effects on bolting time were analysed using a Poisson distribution (R-2.14.1; lmer-package). Significances of explanatory factors were assessed by comparing the minimum adequate model with a reduced model using the likelihood ratio test.

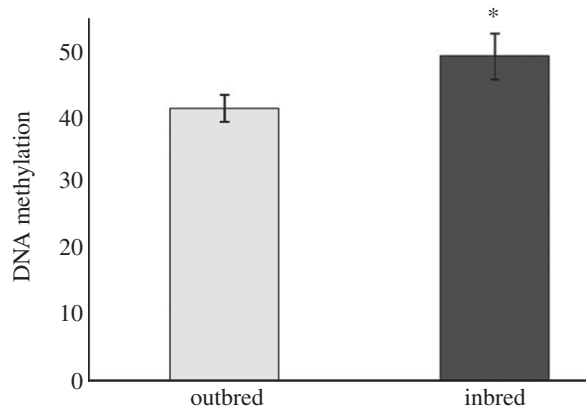
DNA methylation of cytosine was analysed in leaf samples, as a measure of epigenetic variation, using a methylation-sensitive amplification polymorphism (MSAP) technique modified after Keyte *et al.* [12]. MSAP analyses were performed on 200 ng gDNA on a subset of 24 plants (in duplo) using six selective primer combinations (*EcoRI*-AAC/ACA+*MspI*/*HpaII*-TAC/TCA, *EcoRI*-ACA+*MspI*/*HpaII*-TAG, *EcoRI*-AAC+*MspI*/*HpaII*-TGC) on a Beckman CEQ8800 sequencer (Beckman Coulter). Fragments were scored as 'non-methylated' (fragment in *EcoRI*-*HpaII* and *EcoRI*-*MspI*) or 'methylated' (fragment in *EcoRI*-*HpaII* or *EcoRI*-*MspI*) using GENEMARKER (SoftGenetics) (see electronic supplementary materials).

3. RESULTS

As expected [24], F₁ inbred plants showed a reduced performance. Inbreeding depression was observed for leaf number, biomass and photosynthetic efficiency, but not for bolting time (table 1). In outbred plants, a

Table 1. Inbreeding depression in *Scabiosa columbaria*. Inbreeding depression coefficients in different plant traits. Positive numbers indicate inbreeding depression.

	inbreeding depression coefficient		statistics		
	mean \pm s.e.m.		$t_{\text{two-tailed}}$	n_{families}	p -value
leaf number	0.23 \pm 0.04		5.11	6	0.004
biomass	0.46 \pm 0.05		9.40	6	<0.001
photosynthetic efficiency	0.25 \pm 0.04		6.15	6	0.002
bolting time	0.11 \pm 0.27		0.81	4	0.476

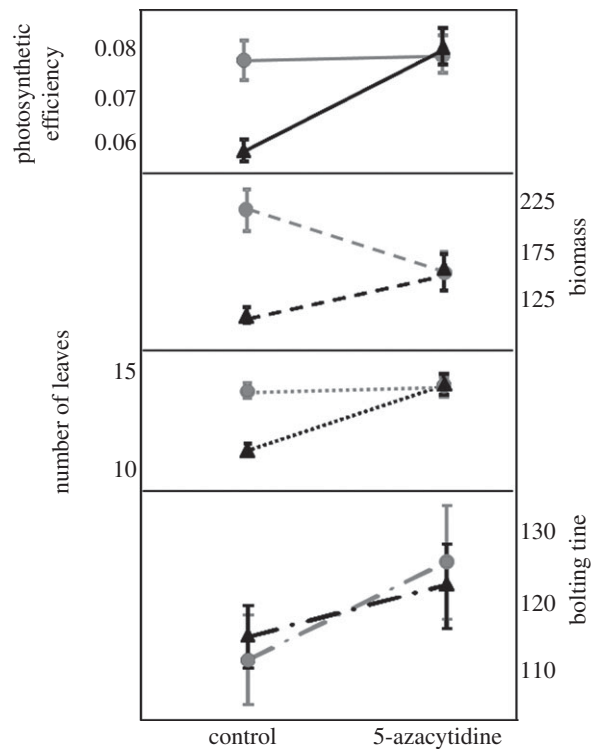
Figure 1. Epigenetic variation in outbred and inbred plants. Percentage of DNA methylation (mean \pm s.e.m.), estimated by the percentage of methylated fragments in 198 MSAP-fragments.

mean methylation percentage of 42 per cent (range 25–60%) was found, similar to the percentages observed in other plant species [12,25,26]. Methylation levels of inbred plants, however, were higher by almost 10 per cent compared with outbred plants ($F_{1,19} = 6.36$, $p = 0.021$; figure 1). 5-Azacytidine reduced DNA methylation in *S. columbaria* by 11 per cent, measured on a separate group of similar-aged plants and grown under similar conditions to the plants in our experiment ($F_{1,22} = 8.11$, $p = 0.009$), comparable with the reduction observed in other species [22,23].

Treatment with 5-azacytidine reduced the mean values of some, but not all, traits in outbred plants. Demethylated outbred plants were on average 29 per cent smaller and bolted 10 days later when compared with control outbred plants (figure 2). Intriguingly, while 5-azacytidine decreased biomass in outbred plants, it increased the average biomass of inbred plants (table 2). In addition, partial demethylation did not affect photosynthetic efficiency and leaf number of outbred plants, but it restored the inbred trait values to the level of the control outbred plants (figure 2). Although biomass in demethylated inbred plants was not completely restored to the level of control outbred plants (figure 2), partial demethylation did significantly increase biomass of inbred plants compared with control inbred plants ($F_{1,28} = 6.65$, $p = 0.016$).

4. DISCUSSION

Strong phenotypic effects were observed in response to experimental demethylation, consistent with observed effects of demethylation in other studies [22,27].

Figure 2. Effects of demethylation on different plant traits. Photosynthetic efficiency (solid lines), biomass (dashed lines), leaf number (square-dotted lines), and bolting time (long-dashed-dotted lines) of outbred (circles with grey lines) and inbred (triangles with black lines) plants treated with and without 5-azacytidine (mean \pm s.e.m.). For statistical results, see table 2.

Intriguingly, our results show a direct effect of inbreeding on epigenetic markers: Inbreeding resulted not only in inbreeding depression for fitness-related traits, but also increased DNA methylation. Most interestingly, when the increased methylation level in inbreds was restored to the outbred level, inbreeding depression was completely (photosynthetic efficiency, leaf number) or almost completely (biomass) eliminated. This strongly suggests that in these cases DNA methylation mediates the negative effects of inbreeding. This suggestion is further enhanced by the observation that, in a trait without inbreeding depression (bolting time), demethylation did not change relative performance. These results underline the potentially important role of DNA methylation in determining the level of inbreeding depression. At the same time, the incomplete restoration of biomass demonstrates that other genetic and/or epigenetic factors contribute to inbreeding effects.

In addition, the observed link between epigenetic variation and inbreeding depression suggests that

Table 2. Summary of mixed effect models. Effects of crossing type, demethylation and their interaction on different plant traits. d.f._{1,2}: degrees of freedom of explanatory factor and error term, respectively. Significant values ($\alpha = 0.05$) are indicated in bold (see electronic supplementary materials).

	d.f. _{1,2}	crossing		demethylation		crossing \times demethylation	
		<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value	<i>F</i>	<i>p</i> -value
leaf number	1,64	2.307	0.134	7.162	0.009	3.993	0.050
biomass	1,64	11.982	0.001	0.102	0.751	10.229	0.002
photosynthetic efficiency	1,64	6.534	0.013	11.199	0.001	8.672	0.005
bolting time ^a		1.247	0.264	29.504	<0.001	2.474	0.116

^aFor bolting time, χ^2 -values were shown (see §2).

environment-dependent inbreeding depression may at least partly be explained via epigenetic processes, providing a new explanation for the interaction between environment and inbreeding depression. Since this experiment was conducted in a single environment, we are not able to distinguish plasticity effects from conditionally expressed alleles and fitness [6]. Our results inspire further research on this.

To our knowledge, there is no clear-cut mechanistic explanation for the observed interplay between epigenetic variation, inbreeding and inbreeding depression. It has been suggested that the increased homozygosity that results from inbreeding may disrupt epigenetic crosstalk between alleles at the same locus [16,17]. This could cause partial inappropriate silencing of genes and consequently result in inbreeding depression. In mammals, for example, one of the two copies of the X-chromosome in females is silenced. This inactivation is regulated epigenetically [28]. Another potential explanation may be that inbreeding disrupts the enzymatic machinery involved in the induction and maintenance of cytosine methylation. This machinery builds on complex genomic and transcriptomic interactions in which methylation maintenance enzymes such as MET1 and CMT3, enzymes for de novo methylation of cytosines such as domains-rearranged methyltransferase (DRM), and regulation via small RNAs and DICER-like enzymes are involved [29]. In addition, interactions with histone modifications are thought to play a role [29]. If deleterious recessive alleles are present at loci that code for enzymes involved in this machinery, inbreeding would result in non-adaptive methylation patterns. However, at present it remains unknown whether a possible disruption of the enzymatic epigenetic machinery can lead to increased methylation as a consequence of inbreeding, and whether this is compatible with the observation that different traits respond differently to inbreeding.

Our results provide new insights into the mechanisms of inbreeding effects. The relative importance of epigenetic compared with genetic effects, however, needs to be tested in future studies. Other studies have shown that Mendelian factors such as recessive deleterious alleles contribute to inbreeding depression (see references in [16]). In addition, other species, such as *Drosophila*, show high levels of inbreeding depression without significant methylation of their DNA ([30], but see [31]). In these species, genetic or other epigenetic processes [17] may contribute more to inbreeding effects.

To conclude, in addition to genetic effects that are known to contribute strongly to inbreeding depression, our results provide strong evidence that epigenetic processes may play an important role.

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