Natural epigenetic variation contributes to heritable flowering divergence in a widespread asexual dandelion lineage

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Abstract

Epigenetic variation has been proposed to contribute to the success of asexual plants, either as a contributor to phenotypic plasticity or by enabling transient adaptation via selection on transgenerationally stable, but reversible, epialleles. While recent studies in experimental plant populations have shown the potential for epigenetic mechanisms to contribute to adaptive phenotypes, it remains unknown if heritable variation in ecologically relevant traits is at least partially epigenetically determined in natural populations. Here, we tested the hypothesis that DNA methylation variation contributes to heritable differences in flowering time within a single widespread apomictic clonal lineage of the common dandelion (*Taraxacum officinale* s. lat.). Apomictic clone members of the same apomictic lineage collected from different field sites showed heritable differences in flowering time, which was correlated with inherited differences in methylation-sensitive AFLP marker profiles. Differences in flowering between apomictic clone members were significantly reduced after *in vivo* de-methylation using the DNA methyltransferase inhibitor zebularine. This synchronization of flowering times suggests that flowering time divergence within an apomictic lineage was mediated by differences in DNA methylation. While the underlying basis of the methylation polymorphism at functional flowering time-affecting loci remains to be demonstrated, our study shows that epigenetic variation contributes to heritable phenotypic divergence in ecologically relevant traits in natural plant populations. This result also suggests that epigenetic mechanisms can facilitate adaptive divergence within genetically uniform asexual lineages.

Introduction

Maintaining high fitness in the face of environmental heterogeneity can be achieved through phenotypic plasticity at the individual level and through genetically based adaptation at the population level. In asexually reproducing organisms, however, adaptation within a clonal lineage is constrained by limited genetic variation due to the absence of recombination and segregation. Asexuals are often considered to be evolutionary dead ends due to presumed limited adaptive capacity and because of the accumulation of deleterious mutations (Lynch *et al.* 1993). Yet, asexual lineages often become extremely successful and widespread after they are introduced into new environments (Zhang *et al.* 2010), suggesting high phenotypic plasticity and/or adaptive potential despite limited genetic variation. Understanding the mechanisms of rapid adaptive responses is relevant as species increasingly face changing biotic and abiotic environments due to global change pressures such as climate change-induced range shifting (Chen *et al.* 2011) and biological invasions (Prentis *et al.* 2008).

Epigenetic inheritance has the potential to contribute to quick adaptive dynamics by allowing heritable yet reversible and potentially environment-sensitive phenotypic variants (Bossdorf *et al.* 2008; Rapp & Wendel 2005). Epigenetics refers to molecular mechanisms that can cause mitotically or meiotically heritable variation in gene activity without changes in the underlying DNA sequence (Bird & Macleod 2004; Richards 2006). Epigenetic mechanisms, of which DNA methylation is probably the most studied, play an important role in various processes including development, environmental responses and transposable element silencing (Chinnusamy & Zhu 2009; Law & Jacobsen 2010; Okano *et al.* 1999). In addition to a role in phenotypic plasticity (Bossdorf *et al.* 2008; Zhang *et al.* 2013) DNA methylation is relevant for ecology and evolution because modified DNA methylation patterns can be inherited over multiple generations (Jablonka & Raz 2009) which creates the opportunity to affect selection and adaptation (Bossdorf *et al.* 2008). A potential to affect adaptation seems particularly high for plants that, compared to mammals, experience less DNA methylation resetting between generations (Feng *et al.* 2010). In experimental *Arabidopsis* populations it was recently shown that artificially induced variation in DNA methylation, in absence of contributions from DNA sequence variation, can control heritable phenotypes for multiple generations (Cortijo *et al.* 2014). Such heritable variation could be especially relevant for the adaptive capacity of asexually reproducing lineages (Douhovnikoff & Dodd 2015; Verhoeven & Preite 2014).

In natural populations, DNA methylation has been proposed to affect adaptation by permitting heritable but transient responses to environmental factors (Verhoeven *et al.* 2010; Zhang *et al.* 2013) or by selecting stable epigenetically determined traits (Kalisz & Purugganan 2004). Abundant variation in DNA methylation can be induced by environments (Dowen *et al.* 2012) and can arise spontaneously (Becker *et al.* 2011; Schmitz *et al.* 2011; van der Graaf *et al.* 2015). Heritable methylation variation that affects phenotypic traits is present in natural populations and methylation patterns show intriguing associations with environments, phenotypes, and populations (Cubas *et al.* 1999; Herrera & Bazaga 2010; Richards *et al.* 2012). These observations support the potential of epigenetic variation to affect plant adaptation. However, beyond such correlations, convincing demonstrations of an adaptive role in nature have remained elusive.

A challenging aspect is to unravel if functional epigenetic variants are autonomous or under genetic control (Dubin *et al.* 2015; Richards 2006). Genetic mutations can exert their functional effects by determining DNA methylation in cis or in trans (Dubin *et al.* 2015). In such cases

DNA methylation is part of the ‘phenotype’ of genetic mutations, and any relevant selection or adaptation takes place at these genetic mutations. However, transgenerationally stable DNA methylation variants can also arise spontaneously, in absence of genetic control (Cubas *et al.* 1999; van der Graaf *et al.* 2015). Such autonomous epigenetic variation has the potential to affect adaptation beyond what genetic variation can explain. In an effort to disentangle autonomous epigenetic variation from the variation introduced by underlying genetic sequence, epigenetic variation is often studied in systems with little or no genetic variation such as completely inbred sexual genotypes or within asexual lineages. Some previous work has suggested adaptive divergence within asexual plant lineages (Turesson 1943) which could be driven by epigenetic differentiation in response to different natural habitats (e.g. Richards *et al.* 2012). However, to date, ecological epigenetic studies are often based on genome-wide patterns at anonymous methylation marker loci, and provide limited insight into the functional epigenetic contribution to adaptation.

Here we investigated the epigenetic contribution to heritable variation in flowering time between plants from the same geographically widespread asexual lineage of the common dandelion (*Taraxacum officinale* L. s. lat.; Asteraceae-Cichorieae, *Taraxacum* sect. *Taraxacum*). Flowering time is an important trait in local adaptation of plant populations, both in response to latitudinal differences (Colautti & Barrett 2013) and to changing local climate (Franks *et al.* 2007). Using a combination of MS-AFLPs and chemical *in vivo* hypomethylation we tested the hypothesis that heritable flowering time variation between apomictic clone members is caused by DNA-methylation divergence within the apomictic lineage.

Methods

Plant material

Asexual genotypes of the common dandelion are typically triploid apomicts that arise from sexual diploid mother plants in mixed sexual-apomict populations via hybridization with apomictic pollen donors (Van Dijk *et al.* 2003). Asexual reproduction in *T. officinale* is through meiotic diplosporous apomixis that involves parthenogenetic embryo formation from unreduced egg cells (Koltunow 1993) resulting in clonal seeds. Dispersal of many different apomictic genotypes from their south-central European areas of origin into northern Europe has occurred since the last ice age (Richards 1973) and some apomictic genotypes have become geographically widespread. One widespread triploid apomict, recognized by taxonomists as microspecies *T. alatum* H. Lindb., was used for this study. Seeds of 14 different *alatum* plants (hereafter called accessions; see Table S1) were collected from fields in Czech Republic,

Germany and Finland in 2012 by expert taxonomists who can recognize this specific apomict from a combination of morphological traits (Kirschner *et al.* in press).

Genetic analysis

For all 14 accessions we grew plants to determine ploidy level and to genotype with microsatellite markers. We determined ploidy level with flow cytometry after DAPI staining using a Partec flow cytometer, comparing each sample to a reference individual of known diploidy (Tas & Van Dijk 1999). All samples were confirmed to be triploid, and thus apomictic (Tas & Van Dijk 1999) (Table S2).

DNA isolation: we isolated total DNA using the hexadecyl-trimethyl-ammonium-bromide (CTAB) procedure described by Rogstad (Rogstad 1992), with minor modifications as described by Vijverberg *et al.* (2004). Briefly, 1.2 cm² fresh leaf tissue was ground with 2 steel balls in 500 µl CTAB-buffer (containing: 100mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB, 1% PVP 40, 1% 2-mercaptoethanol) for 8 minutes using the TissueLyser II (Qiagen, Venlo, The Netherlands). Subsequently, chloroform-isopropanol extraction (24:1, Sigma Aldrich, Zwijndrecht, The Netherlands) and 2-propanol precipitation (Sigma Aldrich, Zwijndrecht, The Netherlands) were performed. RNase treatment was performed for 2 hours at 37°C using 20 mg/ml RNase A (Sigma Aldrich, Zwijndrecht, The Netherlands) dissolved in TE (10 mM Tris (pH8.0), 1 mM EDTA (pH 8.0)). After a final ethanol precipitation and wash steps DNA pellets were dissolved in 50-100 µl TE. We examined DNA quality and quantity using the NanoDrop[®] 2000 spectrophotometer.

Microsatellite analysis: To confirm that the collected accessions were clone members of the same apomictic lineage, we characterized plants of all 14 accessions at a panel of 8 microsatellite loci that are known to be highly polymorphic between different *T. officinale* genotypes (Falque *et al.* 1998; Vašut *et al.* 2004) (in two multiplex PCR reactions; see Table S3). PCR reactions were performed using QIAGEN[®] Multiplex PCR mix, 200 nM of each primer and 30-50 ng DNA in a final volume of 10 µl. The PCR-reaction started with an initial 15 min at 95°C, followed by 30 cycles 30s 94°C; 90s 57°C; 60s 72°C and finished with 30 min at 60°C. PCR products were analysed on an ABI genetic analyser 3130 (Life technologies, Europe BV, Bleiswijk, The Netherlands). We analysed ABI output data using Genemapper v4.0 (Life technologies, Europe BV, Bleiswijk, The Netherlands).

Methylation-sensitive AFLP (MS-AFLP): Prior to the vernalization treatment in the initial flowering time screening experiment (see below), we collected tissue for DNA-isolation from a standardized leaf in two plants per accession. Based on DNA quality we selected eight accessions for MS-AFLP analysis. MS-AFLP analyses were performed with two technical replicates per sample, using the same DNA extraction for two independent MS-AFLP reactions. We followed The MS-AFLP protocol of Keyte *et al.* (2006) with some modifications. DNA was digested with two enzyme combinations: 1) *EcoRI* and *MspI* (containing 10U *EcoRI*, 10U *MspI* (both NEB, Ipswich, United Kingdom), NEB buffer 4 and 50 ng DNA) and 2) *EcoRI* and *HpaII* (containing 10U *HpaII* instead of *MspI*) for 3 h at 37° C in a total volume of 20 µl. *MspI* and *HpaII* both cut 5'-CCGG-3' sequences, but differ in their sensitivity to methylation of the cytosines in these sequences. Both enzymes cleave a non-methylated restriction site. *MspI* also cleaves when the inner cytosine is hemi or fully methylated, but not with the outer cytosine being hemi or fully methylated; *HpaII* only cleaves when the outer cytosine is hemimethylated (Fulnecek & Kovarik 2014). After digestion the adapters were ligated (18 h 22° C, 10 min 65° C) using 1x T4 DNA-ligase buffer (Thermo Fisher Scientific Inc, Landsmeer, The Netherlands), 3.75 pmol *EcoRI* and 37.5 pmol *MspI/HpaII* adapters (Table S4), 1 U T4 DNA-ligase (Thermo Fisher Scientific Inc, Landsmeer, The Netherlands) in a total volume of 30 µl. Subsequent pre-amplification was performed with the pre-selective primers *EcoRI* + A and *MspI/HpaII* + T (See Table S4) (containing 1 x NH₄ buffer, 125 nmol MgCl₂, 2.5 U Taq DNA polymerase (all from GC biotech BV, Alphen aan den Rijn, The Netherlands), 15 pmol of each pre-selective primer (Table S4), 10 nmol dNTPs (Thermo Fisher Scientific Inc, Landsmeer, The Netherlands), and 10 µl of 6.66x diluted ligation product) in a total volume of 50µl (PCR protocol: 2 min 72° C, 20x (30 sec 94° C, 30 sec 56° C, 2 min 72° C), 10 min 60° C). For selective amplification we used eight different selective primer combinations containing a fluorescently labeled *EcoRI* primer to enable pooling of the PCR-products. PCR-reactions were performed in a total volume of 25 µl (containing 1x Taq buffer, 37.5 nmol MgCl₂, 1.25 U Taq DNA polymerase, 7.5 nmol dNTPs, 10µg BSA, 5 pmol labelled selective *EcoRI* primer, 20 pmol selective *HpaII/MspI* primer and 5 µl diluted PCR-product (Table S4) (PCR protocol: 2 min 94° C, 10x (30 sec 94° C, 30 sec 65° C -1° C/cycli, 2min 72° C), 25x (30 sec 94° C, 30 sec 56° C, 2 min 72° C), 10 min 60° C). PCR-products with four different fluorescent labels were pooled, diluted and analyzed using an Applied Biosystems Genetic Analyzer 3130 (Life technologies, Bleiswijk, The Netherlands).

We scored MS-AFLP markers using Genemapper v4.0 (Life Technologies, Bleiswijk, The Netherlands). We scored markers as present when peak intensity exceeded 30 and also

exceeded a locus-specific threshold of average peak intensity across all samples minus two standard deviations of the mean. We discarded all loci showing a signal in a negative control, as well as singleton loci. Some loci showed high inconsistency rates between technical replicates; we discarded such unreliably scored loci if scores were inconsistent between technical replicates in more than 25% of the samples in either *HpaII* or *MspI* profiles. For retained loci, we treated samples with inconsistent scores between technical replicates as missing data. The final data set included 177 *HpaII* markers and 157 *MspI* markers, of which 30 (*HpaII*) and 8 (*MspI*) were polymorphic. The higher polymorphism rate in *HpaII* is consistent with methylation-based rather than genetic variation, as *HpaII* is more sensitive than *MspI* to the CCGG restriction site methylation patterns that are most frequent in plant genomes (Fulnecek & Kovarik 2014). Because interpretation of combined *MspI/HpaII* is known to introduce interpretation ambiguities (Fulnecek & Kovarik 2014) and because of the low rate of *MspI* polymorphisms and the genetic uniformity of apomictic clone members, we further analyzed only *HpaII* profiles and interpreted *HpaII* polymorphisms as methylation polymorphisms. Using a simple matching coefficient we derived a pairwise distance matrix for the 16 samples (PROC DISTANCE in SAS 9.2; dmatch method) which we visualized using a principle coordinate analysis (PCO3 software; Anderson 2004). We tested the accession effect on *HpaII* variation with a multivariate permutation-based test (DISTLM; Anderson 2004) with 10,000 permutations. Based on flowering time line means and multivariate centroid positions of MS-AFLP variation for the eight accessions, we derived distance matrices and tested for correlations between *HpaII* profiles, flowering time and geographic distances. Using exact permutation tests (Zt software; Bonnet & Van de Peer 2002), we tested the correlation between *HpaII* profile and geographic distance with a simple mantel test. We tested correlations between *HpaII* and flowering time (while controlling for geographic distance), and between geographic distance and flowering time (while controlling for methylation) with partial mantel tests.

Flowering time screening

Field-collected achenes of all accessions were sterilized with a five minute wash in 0.5% sodium hypochlorite solution including 0.05% Tween[®]20 (Sigma Aldrich, Zwijndrecht, The Netherlands) followed by a two wash steps of 5 minutes in water, after which they were germinated on 0.8% agar in petri dishes for 7-8 days in a climate cabinet (16/8h light/dark at 20/15°C). Seedlings were transferred individually to 9x9x10cm pots containing 80% potting soil and 20% pumice, and were grown for 38 days in a climate chamber at 14 h light (20°C)

and 10 h dark (15°C), in a randomized complete block design with 5 replicate plants per accession. At day 38 leaves were clipped to 3-4 cm and plants received a four-week vernalization treatment in a cold room at continuous 4°C. After vernalization plants were grown in the same randomized block design in a common greenhouse environment (16 h light, 8 h dark; 20°C, 15°C), with a daylight minimum of 225 $\mu\text{mol}/\text{m}^2/\text{s}$ PAR ensured using additional Son-T GP 600W lights (Philips) at 60% relative humidity. Throughout the experiment, except during vernalization, plants were irrigated two times per week with water and received equal quantities of half-strength Hoagland nutrient solution biweekly. The day of first flowering was scored daily between 10.00 AM and 12.00 PM. Plants were recorded as flowering when the first capitulum opened and yellow petals were visible. Achenes were collected for the de-methylation experiment.

De-methylation experiment

Based on the initial flowering time screening we selected six accessions to evaluate flowering time differences under experimental de-methylation. For each accession, achenes from 3-4 different mother plants from the initial screening experiment were pooled, sterilized and germinated as described above on agar containing 0, 1 or 10 μM zebularine (Sigma Aldrich, Zwijndrecht, The Netherlands). Zebularine is a DNA-methyltransferase inhibitor, related to 5-azacytidine but more stable and causing less negative side-effects, and its application to seedlings results in partial, transient and dose-dependent hypomethylation of the genome, which can cause reactivation of epigenetically silenced loci (Baubec *et al.* 2009). Plants were grown as described above, in a randomized block design with eight replicated blocks in a climate chamber with two plants per accession per block for each zebularine treatment level. After clipping (day 38) half of the plants received a vernalization treatment as described above and the other half was placed directly under greenhouse conditions. After vernalization we also placed these plants in the greenhouse following the original randomized block design as in the climate chamber. First flowering dates were scored as described above. After 13 weeks when flowering had ceased all plants were harvested, dried at 70°C for 4 days, and shoot biomass was determined.

Statistical analysis

Flowering time data in the de-methylation experiment were heteroscedastic (with different variances depending on vernalization treatment and accession) and non-normally distributed because of several late-flowering plants. We therefore analyzed our data using non-parametric

analyses. First, we used a non-parametric anova (PROC NPAR1WAY in SAS 9.2; median score analysis) to test for accession differences in median flowering times. Second, we used a nonparametric survival analysis to compare flowering time distributions between accessions (Vermerris & McIntyre 1999). A survival analysis approach considers not only accession means but also takes into account differences in flowering time variance (flowering synchrony) between accessions and also uses information from plants that failed to flower during the experiment (five non-flowering plants in our experiment). This analysis therefore provides for a more sensitive test of flowering time differences than comparison of means only. In this non-parametric survival analysis each of the six experimental groups (two vernalization treatments x three zebularine levels) were analyzed separately to test for homogeneity of survival curves between the six accessions while correcting for possible block effects (PROC LIFETEST in SAS 9.2). Accession and zebularine effects on biomass were tested using general linear models (PROC GLM in SAS 9.2).

Results

DNA methylation variation between accessions

Microsatellite analysis revealed identical multilocus genotypes for all accessions at a panel of eight microsatellite loci (Table S5), which confirms that the accessions belong to a single asexually reproducing lineage. Despite this genetic similarity, significant DNA methylation variation was detected between accessions (Fig. 1). Variation in methylation-sensitive AFLP profiles based on genomic *Hpa*II digestion was mostly partitioned between accessions (accession effect: multivariate permutation-based test, pseudo- $F_{7,8}=3.2$, $p<0.001$; proportion of variance explained = 0.73; see Fig. 1) and not between replicate plants of the same accession (between-replicate variance, which includes technical error variance, accounted for the remaining proportion = 0.27), indicating stably inherited methylation variation typical for plants (especially in CG contexts; Feng *et al.* 2010). Some differences were found between offspring individuals from the same mother plant, consistent with modest levels of dynamic DNA-methylation.

Flowering time variation between accessions

When raised in a common greenhouse environment, plants grown from field-collected seeds revealed large differences in flowering time between the 14 accessions (accession effect: nonparametric survival analysis test of homogeneity of flowering curves, $\chi^2=37.82$; $p=0.0003$;

see Fig. 2A). No clear latitudinal trend was observed in flowering time: accessions from Finland did not show consistently different flowering than accessions from Czech Republic or Germany. No correlation was observed between *HpaII* variation and geographic distance or between geographic distance and flowering time. However, differences in flowering time showed a stronger correlation with DNA methylation profiles ($r = 0.36$, $p = 0.053$; Fig. 2B), suggesting a potential of DNA methylation to be involved in flowering time differences between the accessions.

Effects of experimental de-methylation

To test the causality between DNA methylation variation and flowering time we examined flowering time of six selected accessions in the subsequent generation after treatment with the DNA-methylation inhibitor zebularine. Because zebularine can cause genomic hypomethylation we hypothesized that it has the potential to nullify trait differences between accessions if these are based on DNA methylation differences. In the absence of zebularine, accession mean flowering times in these second-generation plants were correlated with flowering times in the first experiment (spearman rank correlation coefficient of flowering time after vernalization between experiments: $r = 0.77$) and median flowering times differed significantly between accessions in both the vernalized and in the non-vernalized groups (supporting information S6). This correlation confirms that flowering time differences between accessions are stable and not merely caused by transient maternal effects that might have been associated with the different seed collection field sites.

Survival analysis of flowering time data revealed a significant convergence of flowering time curves due to zebularine treatment (Fig. 3). In the absence of zebularine, differences in flowering time curves were (marginally) significant between accessions, both in the non-vernalized ($\chi^2 = 10.00$, $p = 0.075$) and in the vernalized ($\chi^2 = 23.99$, $p = 0.0002$) group, while in the presence of zebularine these significant differences disappeared (Fig. 3). Zebularine did not cause a consistent shift across accessions towards earlier or later flowering in either the vernalized or the non-vernalized groups; its main effect was to synchronize the flowering curves of the different accessions. Some of the plants flowered strikingly late, but this delayed flowering did not affect the non-parametric survival analysis of flowering time and excluding these late-flowering plants from analysis did not change the conclusions (accession effect in the vernalized group after excluding late-flowering outliers: $p_{\text{zebu0}} = 0.01$; $p_{\text{zebu1}} = 0.41$; $p_{\text{zebu10}} = 0.33$; in the non-vernalized group: $p_{\text{zebu0}} = 0.085$; $p_{\text{zebu1}} = 0.27$; $p_{\text{zebu10}} = 0.53$). Also the non-parametric anova analysis, in both the vernalized and the non-vernalized environments, showed

(marginally) significant differences in accession median flowering times only in the absence of zebularine, but not in the presence of zebularine (supporting information S6). Thus, zebularine acted to synchronize flowering time distributions across the six accessions, nullifying heritable differences in flowering time between these accessions derived from natural populations.

Discussion

We observed that heritable flowering time differences between dandelion apomictic clone members correlate with DNA methylation patterns and are nullified by experimental hypomethylation. These observations demonstrate that DNA methylation mediates heritable flowering time variation within this apomictic dandelion lineage. The potential of DNA methylation polymorphisms to control flowering time variation was previously demonstrated in experimental *Arabidopsis* populations in which artificially induced and stably segregating DNA methylation variation caused flowering differences between isogenic lines, even in the absence of DNA sequence variation (Cortijo *et al.* 2014). Our study shows that heritable variation in flowering time also has an epigenetic component in natural systems.

The notable feature of our experiment is that experimental *in vivo* hypomethylation reduced flowering time differences between accessions, indicating a causal contribution of DNA methylation to flowering time variation. While relatively crude, treatments with demethylation agents provide a useful tool for experimental studies testing the consequences of genomic methylation (Bossdorf *et al.* 2010; Vergeer *et al.* 2012). One possible caveat is that treatment with high doses of DNA methyltransferase inhibitors can lead to detrimental effects on development (Ghoshal & Bai 2007), for instance due to DNA damage (Liu *et al.* 2015). Yet, compared to other demethylating agents such as azacytidine, zebularine has comparatively mild side effects, such as slightly retarded plant growth (Baubec *et al.* 2009). In our study no negative growth effects of zebularine were observed. Instead, we found slightly increased biomass at high zebularine concentration in the non-vernalized group and this effect appeared to be present in some accessions but not in others (supporting information S7). Accessions significantly differed in biomass, and these differences seemed to increase with the highest zebularine concentration (supporting information S7). This variation-inducing effect of zebularine seems to be opposite to the synchronizing effect of zebularine on flowering time. One possible explanation is that responsible genes are inactivated by methylation in all accessions but at different quantitative methylation levels, causing reactivation by the zebularine treatment to

different degrees in different accessions. Alternatively, experimental demethylation may expose the expression of genetic differences between accessions (due to mutation accumulation including transposable element insertions) that are masked by DNA methylation in the natural state.

We did not directly examine the effects of zebularine treatment on genomic DNA methylation. Therefore, we cannot confirm that the zebularine treatment led to strong demethylation. Our study also does not show which genes are involved, as MS-AFLP's do not provide any functional insight in differentially methylated loci; higher-resolution follow up studies based on bisulfite sequencing will be required for that. However, the synchronizing effect of zebularine treatment on flowering time variation implies that functional DNA methylation differences exist between the accessions at one or more loci that affect flowering time. In principle, such methylation differences can have arisen in two different ways. One possibility is that the responsible methylation variation represents a spontaneous and transgenerationally stable epimutation, in which case divergence between apomictic clone members is based on autonomous epigenetic variation (*sensu* Richards 2006). Autonomous DNA methylation variation was recently demonstrated to underlie heritable flowering time variation in an experimental *Arabidopsis* epiRIL population, suggesting a potential of autonomous methylation variation to affect this trait also in nature (Cortijo *et al.* 2014).

Alternatively the functional methylation variation may be controlled by a recent genetic mutation that arose within the apomictic lineage. For instance, a transposition event that occurred near a flowering time locus could attract DNA methylation that silences the new transposable element copy but this silencing can affect also the activity of nearby genes (Slotkin & Martienssen 2007), thus potentially creating epigenetic modification of flowering time loci. Genetically controlled DNA methylation variation at flowering time genes has been observed between genetically divergent *A. thaliana* genotypes (Zhai *et al.* 2008). In such cases epigenetic silencing is the mechanism through which a genetic mutation exerts its diversifying effect on flowering time. By analyzing plants from a single apomictic lineage our study aimed to minimize the effects of genetic variation between accessions, thus predisposing to detect autonomous epigenetic variation. However, genetic mutations accumulate between apomictic clone members and especially transposable element activity can cause differences between accessions (Ferreira de Carvalho *et al.* submitted). Further genomic analysis will be required to either confirm autonomy or to identify genetic control of the methylation variation that is

responsible for the observed flowering time differences and to confirm a de-methylating effect at these loci of the zebularine treatment.

While our study did not aim to demonstrate direct adaptive advantage of flowering time differences, it is known that flowering time is an important trait in adaptation to higher latitudes (Colautti & Barrett 2013) and other ecological gradients. Although there were large latitudinal and longitudinal differences between collection sites, we did not find any consistent geographic gradient in flowering time in either the vernalized first generation (see Fig. 1) or the non-vernalized second generation plants. The lack of such a gradient has also been observed in other studies that traced differences in flowering time to microhabitat variation (Sandring *et al.* 2007; Franks *et al.* 2007) rather than large-scale geographic gradients.

In conclusion, we demonstrate heritable divergence in flowering time within a single apomictic dandelion lineage, mediated by within-lineage divergence of DNA methylation. The epigenetic contribution to adaptation has been intensively discussed, but has remained largely undemonstrated. Our results suggest that heritable methylation variation can be an important contributor to adaptive trait variation in natural populations. While further molecular characterization is necessary to unravel if there is a genetic basis to the observed epigenetic effects and how methylation variation acts on functional genes, our results demonstrate that epigenetic inheritance adds to the adaptive capacity of plant populations: either via autonomous epialleles or as a mechanism through which genetic mutations such as transpositions can realize heritable gene expression differences. Epigenetic variation therefore contributes to the adaptive potential of populations which may be particularly relevant for species that face rapidly changing environments or when genetic variation is limited.

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Data accessibility

Flowering time, microsatellite and MS-AFLP data are deposited in Dryad: Data from: Natural epigenetic variation contributes to heritable flowering divergence in a widespread asexual dandelion lineage, DOI:10.5061/dryad.hs26b.

Authors' contributions statement

RAW carried out the greenhouse experiments, performed the ploidy analysis and contributed to data analysis; RAW and CO performed the microsatellite and MS-AFLP analysis; LBS contributed to data analysis; JK was responsible for seed collections in the field; KJFV designed the study and contributed to data analysis. The paper was written by RAW and KJFV and all authors commented on the manuscript.

Figures

Figure 1. *Taraxacum alatum* accessions show within-lineage divergence in genomewide DNA methylation. Principal Coordinate analysis of pairwise *HpaII*-profile distances of eight accessions from different geographic origins (red: Germany; blue: Czech Republic; green: Finland; see also Table S1) shows that replicate plants from the same accession tend to show similar *HpaII* profiles (accession effect: $p < 0.001$; see text). This indicates heritable DNA methylation differentiation between the accessions.

Figure 2. *Taraxacum alatum* accessions show within-lineage divergence in flowering time that is correlated with DNA methylation. Panel A: Accessions from different geographic origins (red: Germany; blue: Czech Republic; green: Finland; see also Table S1) show significant flowering time differences when grown in a common greenhouse environment (nonparametric survival analysis test of homogeneity of flowering time curves; see methods). Boxes and whiskers represent 25%-75% quartiles and minimum-maximum observations, respectively. Panel B: Flowering time differences are correlated with *HpaII*-profiles, but not with geographic distance. Correlations of pairwise distance matrices were evaluated using mantel tests (DNA methylation versus geographic distance) and partial mantel tests (flowering time versus either DNA methylation or geographic distance while controlling for the other variable).

Figure 3. Flowering time differences between six different *Taraxacum alatum* accessions are nullified by experimental *in vivo* de-methylation. In both non-vernalized plants (left panels) and vernalized plants (right panels), accessions show differences in flowering time under control conditions (top panels), as indicated by significant differences in cumulative flowering curves obtained from eight replicate plants per accession. Exposure during germination to 1 μM (middle panels) or 10 μM (bottom panels) of the DNA methylation inhibitor zebularine leads to converging flowering times. Accession differences in flowering were tested for each treatment group separately using nonparametric survival analysis (see methods).

Supportive information

Table S1 *Collection information of Taraxacum alatum accessions.*

Accession code used in this study, original collection ID, country of origin and coordinates of collection site.

Code	Collection ID	Country	Latitude/Longitude
G1	1	Germany	50° 47' 15" N 13° 51' 22" E
G2	2	Germany	50° 47' 15" N 13° 51' 22" E
G3	1310-S1	Germany	50° 27' 41" N 12° 48' 55" E
G4	1313-S1	Germany	50° 34' 28" N 13° 17' 30" E
CR1	4-2	Czech Republic	50° 02' 32" N 14° 21' 28" E
CR2	1280-S2	Czech Republic	49° 20' 56" N 17° 18' 31" E
CR3	1284-S2	Czech Republic	49° 16' 23" N 17° 58' 01" E
F1	11-101	Finland	62° 37' 42" N 29° 44' 08" E
F2	12-136	Finland	62° 37' 55" N 29° 41' 00" E
F3	12-137	Finland	62° 37' 55" N 29° 41' 00" E
F4	12-153	Finland	62° 13' 34" N 30° 19' 59" E
F5	12-155	Finland	62° 13' 34" N 30° 19' 59" E
F6	12-170	Finland	62° 11' 57" N 30° 35' 51" E
F7	12-173	Finland	62° 11' 57" N 30° 35' 51" E

Table S2 *Taraxacum alatum accessions are triploid.*

Partec flow cytometer output of nuclear DNA content of a diploid reference plant (Peak_{ref}) and each of the *alatum* accessions (Peak_{acc}). Peak ratios ($\text{Peak}_{\text{acc}} / \text{Peak}_{\text{ref}}$) of ≈ 1.5 confirm triploidy of the accessions.

Accession	Peak_{ref}	Peak_{acc}	Peak ratio
G1	117,96	179,51	1,52
G2	118,80	182,10	1,53
G3	121,41	188,67	1,50
G4	120,98	182,90	1,51
CR1	117,40	185,50	1,58
CR2	114,70	175,13	1,53
CR3	115,62	178,93	1,55
F1	122,80	185,80	1,51
F2	119,55	185,27	1,55
F3	117,54	181,55	1,55
F4	118,65	181,27	1,53
F5	119,41	183,06	1,53
F6	115,05	179,53	1,56
F7	118,91	180,71	1,52

Table S3 *Microsatellite characteristics*

Repeat motif, reference (1 = Falque *et al.* (1998); 2 = Vašut *et al.* (2004)) primer sequences, annealing temperature (T_a), product size and multiplex PCR (1 or 2).

Marker	Reference	Repeat	Forward Primer	Reverse Primer	T _a	Size	Mix
MSTA31	1	(CT)17	CCTCAAAGCCCGAACTT	ACGACCCCAACTGATTTTAC	51	240	1
MSTA44B	1	(CT)19	AGTTTCTCTAAATGGGAAGAT	TGTCAGGTATATTCAAAGATTC	51	191	1
MSTA58	1	(CT)17	CCACTTTGAACGACATTGTTA	TCTCTGATTCAACCTGTGTTTC	53	125	1
MSTA61	1	(CT)9(GT)8	CTACGCTCTTCCCCATTGAT	TGGTGGTTGATGCTCTGTTT	55	126	2
MSTA67	1	(TC)22T(CA)12	TTCGGATATGACCCTTCACT	GACATCTTGCACCTAAAAACAAT	56	219	2
MSTA72	1	(CT)18	GATGAATTTGTCTTTGCT	CTATATCATTTTAGGTGTCGT	49	181	2
MSTA78	1	(CT)9	TGATTGATTCTGCCCTAAACC	TGCCAAGACATCCGAAAAG	52	151	1
MSTA143	2	(GA)15	GATTTTCGCCGGTGAGTAGG	TTCCCAACATAAACCAAAAAGATA	55	243	2

Table S4 *MS-AFLP primers*

Sequences of primers and adapters used during ligation, pre-amplification and amplification of MS-AFLP's.

Primer	Sequence 5'-3'
Adapters	
<i>Eco</i> RI adapter I	CTCGTAGACTGCGTACC
<i>Eco</i> RI adapter II	AATTGGTACGCAGTC
<i>Hpa</i> II/ <i>Msp</i> I adapter I	GATCATGAGTCCTGCT
<i>Hpa</i> II/ <i>Msp</i> I adapter II	CGAGCAGGACTCATGA
Preselective primers	
<i>Eco</i> RI+A	GACTGCGTACCAATTCA
<i>Hpa</i> II/ <i>Msp</i> I + T	ATCATGAGTCCTGCTCGGT
Selective primers	
<i>Eco</i> RI+AAC	GACTGCGTACCAATTCAAC
<i>Eco</i> RI+ACA	GACTGCGTACCAATTCACA
<i>Eco</i> RI+AG	GACTGCGTACCAATTCAG
<i>Eco</i> RI+ACC	GACTGCGTACCAATTCACC
<i>Hpa</i> II/ <i>Msp</i> I + TCA	ATCATGAGTCCTGCTCGGTCA
<i>Hpa</i> II/ <i>Msp</i> I + TAC	ATCATGAGTCCTGCTCGGTAC
<i>Hpa</i> II/ <i>Msp</i> I + TAG	ATCATGAGTCCTGCTCGGTAG

Table S5 *Taraxacum alatum* accessions show uniform microsatellite allele sizes
Microsatellite allele peak positions as observed in the ABI output profiles. All 14 accessions show the same alleles on three single-allelic and five multi-allelic microsatellite loci, confirming their clonal relationship. Because of triploidy, either one, two or three alleles are present.

MSAT	Allele	Accession													
		G1	G2	G3	G4	CR1	CR2	CR3	F1	F2	F3	F4	F5	F6	F7
M58	Allele 1	124,7	124,7	124,6	124,6	124,7	124,6	124,6	124,6	124,6	124,6	124,7	124,7	124,6	124,7
M44B	Allele 1	185,6	185,5	185,4	185,4	185,5	185,4	185,4	185,5	185,5	185,5	185,4	185,5	185,4	185,4
M31	Allele 1	238,0	238,0	237,7	237,7	238,1	237,7	237,8	238,0	237,9	237,8	237,8	237,8	237,8	237,7
M78	Allele 1	164,2	164,2	164,1	164,1	164,2	164,0	164,0	164,2	164,1	164,1	164,1	164,2	164,0	164,0
	Allele 2	172,5	172,6	172,3	172,4	172,4	172,3	172,3	172,5	172,4	172,4	172,5	172,4	172,4	172,3
M61	Allele 1	136,1	136,0	136,1	136,1	136,1	136,1	136,1	136,1	136,1	136,1	136,1	136,1	136,1	136,1
	Allele 2	138,2	138,1	138,2	138,2	138,2	138,2	138,2	138,2	138,2	138,2	138,3	138,2	138,2	138,2
M67	Allele 1	203,2	203,1	203,2	203,2	203,2	203,2	203,2	203,2	203,2	203,1	203,3	203,2	203,3	203,2
	Allele 2	221,7	221,6	221,7	221,7	221,7	221,7	221,7	221,7	221,7	221,7	221,7	221,8	221,7	221,7
M72	Allele 1	175,0	175,0	175,0	175,0	175,0	175,1	175,0	175,0	175,0	174,9	175,0	175,0	175,0	175,0
	Allele 2	208,9	208,8	208,8	208,8	208,8	208,8	208,9	208,8	208,7	208,7	208,9	208,8	208,9	208,7
M143	Allele 1	238,1	238,2	238,2	238,2	238,2	238,2	238,2	238,3	238,1	238,2	238,3	238,2	238,2	238,1
	Allele 2	240,1	240,1	240,1	240,1	240,1	240,1	240,1	240,1	240,1	240,1	240,1	240,1	240,1	240,1
	Allele 3	245,6	245,7	245,7	245,6	245,6	245,6	245,6	245,7	245,7	245,7	245,7	245,7	245,7	245,6

Supporting information S6: Non-parametric ANOVA on *T. alatum* accession flowering time medians

Figure S6: Demethylation effect on flowering time medians of *T. alatum* accessions.
Zebularine treatment leads to homogenization of flowering time in the high zebularine concentration in the non-vernalized group and in the low zebularine concentration in the vernalized group (Also see Table S6).

Factor	Vernalization	Zebu	test	p-value
accession	not	0	median	0.068
	not	1		0.29
	not	10		0.29
	yes	0		0.042
	yes	1		0.47
	yes	10		0.39

Table S6: Non-parametric linear model on flowering time medians.

Using a non-parametric one-way ANOVA, between-accession differences of the median flowering time were tested per zebularine treatment group. Both in the vernalized and non-vernalized group, (marginally) significant differences in flowering time median were found. These differences disappeared in the zebularine-treated plants.

Supportive information S7 Zebularine does not negatively affects plant performance.

We tested the effect of zebularine treatment and vernalization on ln-transformed shoot biomass with a general linear model (see table S7). This revealed significant accession differences and pointed at a slightly increased biomass in plants treated with zebularine. However, individual accessions reacted in a specific way to zebularine treatment (see figure S7).

Figure S7: Final shoot biomass (g) of six different *Taraxacum alatum* accessions under vernalized and non-vernalized conditions, after exposure to 0, 1 or 10 μ M zebularine during germination.

Table S7: ANOVA table on the main and interaction effects of zebularine and vernalization treatment on the biomass of 6 *T. alatum* accessions.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
block	7	4,93	0,70	21,9	<0,0001
Accession	5	1,45	0,29	8,99	<0,0001
Zebularine	2	0,22	0,11	3,49	0,0321
Accession*Zebularine	10	0,72	0,07	2,25	0,0161
Vernalization	1	0,33	0,33	10,35	0,0015
Accession*Vernalization	5	0,10	0,02	0,63	0,6795
Zebularine*Vernalization	2	0,01	0,00	0,14	0,8703
Accession*Zebularine*Vernalization	10	0,25	0,03	0,78	0,6502