MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 4406–4418 doi: 10.1111/mec.13329

The epigenetic footprint of poleward range-expanding plants in apomictic dandelions

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Abstract

Epigenetic modifications, such as DNA methylation variation, can generate heritable phenotypic variation independent of the underlying genetic code. However, epigenetic variation in natural plant populations is poorly documented and little understood. Here, we test whether northward range expansion of obligate apomicts of the common dandelion (*Taraxacum officinale*) is associated with DNA methylation variation. We characterized and compared patterns of genetic and DNA methylation variation in greenhouse-reared offspring of *T. officinale* that were collected along a latitudinal transect of northward range expansion in Europe. Genetic AFLP and epigenetic MS-AFLP markers revealed high levels of local diversity and modest but significant heritable differentiation between sampling locations and between the southern, central and northern regions of the transect. Patterns of genetic and epigenetic variation were significantly correlated, reflecting the genetic control over epigenetic variation and/or the accumulation of lineage-specific spontaneous epimutations, which may be selectively neutral. In addition, we identified a small component of DNA methylation differentiation along the transect that is independent of genetic variation. This epigenetic differentiation might reflect environment-specific induction or, in case the DNA methylation variation affects relevant traits and fitness, selection of heritable DNA methylation variants. Such generated epigenetic variants might contribute to the adaptive capacity of individual asexual lineages under changing environments. Our results highlight the potential of heritable DNA methylation variation to contribute to population differentiation along ecological gradients. Further studies are needed using higher resolution methods to understand the functional significance of such natural occurring epigenetic differentiation.

Keywords: apomictic dandelions, DNA methylation, epigenetic inheritance, MS-AFLP, population epigenetics, range expansion

Received 26 July 2014; revision received 10 July 2015; accepted 15 July 2015

Introduction

Plant species have the ability to respond to a changing climate by phenotypic plasticity, adaptation and migration towards more suitable habitats (Nicotra *et al*. 2010). In practice, these processes are intermingled; for example, adaptive changes may arise during migration. At the leading edge of the migration front during range expansion, plants have to adapt to novel biotic and abiotic conditions (Davis & Shaw 2001). Numerous asexual plant species consist of individual clonal genotypes of which many have successfully colonized a wide range of new habitats (Hollingsworth & Bailey 2000; Ahmad *et al*. 2008; Zhang *et al*. 2010). Because of their limited within-lineage genetic variation, asexuals largely rely on the capacity of phenotypic plasticity to cope with new environmental conditions (Castonguay & Angers

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2012). An important question is to determine what factors enable asexuals to successfully persist in changing climatic conditions.

Recent findings suggest that epigenetic mechanisms, such as histone modification and DNA methylation, may represent an additional source of phenotypic variation that is relevant to both within-generation phenotypic plasticity and, if stably transmitted, also heritable adaptation (Angers *et al.* 2010). Epigenetic mechanisms play an important role in regulating gene expression and stabilizing the genome by repressing harmful genetic elements, such as transposable elements (Henderson & Jacobsen 2007). In plants, DNA methylation variation also shows substantial heritability (Cervera *et al.* 2002; Anway *et al.* 2005; Jablonka & Raz 2009; Johannes *et al.* 2009; Cortijo *et al.* 2014). Without changing the genetic code, epigenetic mechanisms can generate stable phenotypic variation contributing to phenotypic plasticity both in sexual (Bossdorf *et al.* 2010; Zhang *et al.* 2013) and in asexual species (Angers *et al.* 2010; Latzel & Klimešová 2010). In particular, in plants, stable DNA methylation variation can account for heritable trait differences that persist for multiple generations (Cubas *et al.* 1999; Cortijo *et al.* 2014). Variation in epigenetics may not only arise spontaneously (Becker *et al.* 2011; Schmitz & Ecker 2012) but may also be environmentally induced (Verhoeven *et al.* 2010b; Dowen *et al.* 2012; Sahu *et al.* 2013).

The current knowledge of epigenetic variation derives mainly from studies using model species under controlled conditions. It has been only recently that studies have started to focus on patterns of DNA methylation variation in natural systems, in order to understand the evolutionary and ecological role of epigenetics (Bossdorf *et al.* 2008; Richards 2008; Bossdorf & Zhang 2011). Studies in natural plant populations have shown that DNA methylation variation can be correlated with ecological stresses (Herrera & Bazaga 2011) and habitats (Gao *et al.* 2010; Lira-Medeiros *et al.* 2010; Paun *et al.* 2010). Further, common garden studies on the clonally reproducing and invasive Japanese knotweed revealed habitat differentiation by DNA methylation and only limited genetic variation (Richards *et al.* 2012). These findings suggest that epigenetic variation may enable individual asexual lineages to adapt under changing environments.

The focus of this study is on epigenetic variation during range expansion in asexual plant species. We compare patterns of genetic and heritable DNA methylation variation in natural populations of apomictic dandelions (*Taraxacum officinale*) along a geographic transect of their range expansion. *Taraxacum officinale* reproduces sexually or asexually via apomixis, that is production of nonfertilized seeds (Asker & Jerling 1992), and in Europe, it shows a pattern of geographic parthenogenesis reflecting postglacial range expansion (Menken *et al.* 1995; Verduijn *et al.* 2004). After the last retreat of land ice, approx. 10 000 years ago obligate apomicts migrated from glacial refugia towards northern Europe (Comes & Kadereit 1998). Triploid apomictic lineages co-occur with diploid sexually reproducing dandelions in south central Europe, the area of the glacial refugia, and new apomictic (triploid) lineages arise through hybridization between sexual mothers and apomictic pollen donors (Richards 1973; Mogie & Ford 1988; Tas & Van Dijk 1999). It is believed that apomictic lineages are continuously formed in these mixed populations, and together with the northward migration, this process may account for the high levels of clonal diversity typically observed in northern European populations of apomictic dandelions (Van der Hulst *et al.* 2001). Previous work on apomictic dandelions showed that heritable DNA methylation changes can be triggered by exposure to ecological stresses (Verhoeven *et al.* 2010b) and by the hybridization of sexual and asexual dandelions, which gives rise to novel apomictic plants (Verhoeven *et al.* 2010a).

Heritable epigenetic modifications can be functionally targeted (for instance when a specific environmental cue triggers a specific epigenetic modification) or essentially random (Shea *et al.* 2011) and range from transient to very stable across generations (Becker *et al.* 2011; Cortijo *et al.* 2014). Targeted epigenetic effects could function as an underlying mechanism for specific stress responses, inherited stress 'memory' and transgenerational phenotypic plasticity. By contrast, random epigenetic variation, if stably inherited, could function as a basis for natural selection on epimutations (Hirsch *et al.* 2012). Environmental stress, such as exposure to novel habitats during range expansion, can change DNA methylations, histone modifications, transposon silencing and gene expression, which subsequently generates random and novel genetic and epigenetic variation (Rapp & Wendel 2005; Bilichak *et al.* 2012). In that case, we would expect that DNA methylation variation is increased in apomictic dandelions of northern regions due to their history of encountering novel biotic and abiotic environments during their northward range expansion. We would also expect DNA methylation variation to differentiate along the geographic transect. As environmental conditions change, DNA methylations can specifically be modified and can result in different epigenetic patterns associated with the habitats along the range expansion gradient. In addition, selection acting on random but stable epimutations may also contribute to differentiation between habitats in the DNA methylation profile.

Here, we studied epigenetic variation along a geographic transect of northward migration of apomictic dandelion in north western Europe. We used offspring from field-derived plants to analyse the heritable component of DNA methylation variation. With this experimental design, the captured heritable component of DNA methylation variation potentially includes both DNA methylation polymorphisms that are stably transmitted for many generations (e.g. Cortijo *et al.* 2014) and possible environmentally induced methylation modifications associated with the maternal growing environment (Verhoeven *et al.* 2010b). Specifically, we tested the following hypotheses: (i) northern populations show higher levels of DNA methylation variability than southern populations. Such a pattern could arise because of higher levels of stress-induced DNA methylation modifications in the lineages' novel northern environments. (ii) Regions along the transect are epigenetically differentiated. This could arise from environment-specific DNA methylation patterns. (iii) DNA methylation variation patterns are partly autonomous, that is independent of underlying genetic variation. DNA methylation variation can be controlled by, or act independent of, underlying genetic polymorphisms (Richards 2006). A relevant issue is therefore the degree of independence from the underlying genetic code in epigenetic variation. Many of the known epialleles are associated with silencing of transposable elements that can affect the expression of nearby genes (Paszkowski & Grossniklaus 2011). However, some features of the DNA methylome show autonomous variation independent of genetic variation (Cubas *et al.* 1999; Kalisz & Purugganan 2004; Marfil *et al.* 2012; Schmitz *et al.* 2013). Sequence-independent epialleles can potentially allow for adaptive dynamics that cannot be explained by the genetic code alone (Bossdorf *et al.* 2008), which may be particularly relevant in asexuals since they have limited within-lineage genetic variation (Castonguay & Angers 2012; Verhoeven & Preite 2014).

Materials and methods

Study species and sampling design

The common dandelion, *Taraxacum officinale*, is a widespread perennial plant species that is dispersed through seeds. For the description of the taxon *T. officinale*, formerly grouped in the sections *Vulgaria* and *Ruderalia*, see Kirschner & Štěpánek (2011). In spring 2011, we collected seeds from apomictic dandelions in ten areas (which we refer to as populations) along a south–north transect from Luxembourg to central Sweden (Fig. 1). This transect covers a large portion of the apomicts' distribution in north western Europe. The southernmost part of the transect is situated close to the area of mixed sexual–asexual populations in south central Europe where new apomicts can arise from sexual ancestors (Menken *et al.* 1995; Verduijn *et al.* 2004). Within each population, we collected one seed head from each of 16 different fields within an approximate 5–10 km radius to obtain an unbiased sample of the genetic diversity of the local population (Table 1). Sampling localities were usually pastures and some fallows, roadsides and forest glades. From each field-collected seed head, we grew one offspring. Seeds were germinated on moist filter paper in Petri dishes for 11–16 days (10-h dark: 14-h light; 15: 20 °C). Individual seedlings were transplanted into 1-L pots containing a mixture of 80% potting soil and 20% pumice. The plants were grown for 3 months in a fully randomized design in the greenhouse (8-h dark: 16-h light; 16: 21 °C) and watered several times per week, depending on the rate of water loss. In addition, plants received 50 mL of half-strength Hoagland

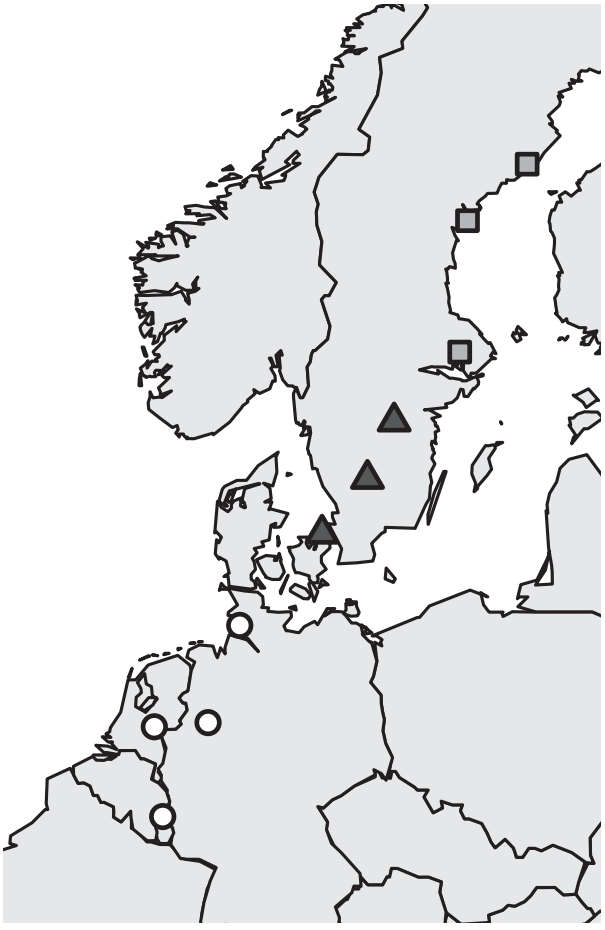

Fig. 1 Sampling localities grouped in three regions indicated by the following: white circle, south; black triangle, centre; and grey rectangle, north. For further description of the localities, see Table 1.

Table 1 Overview of the sampled apomictic dandelions

ID	Region	Population	Latitude	Longitude
N_3	North	Umeå, SE	63°49′33.06″N	20°15′46.94″E
N_2	North	Söderrå, SE	62°37′56.17″N	17°56′27.13″E
N_1	North	Uppsala, SE	59°51′30.82″N	17°38′20.15″E
C_3	Centre	Skänninge, SE	58°23′42.97″N	15°05′11.80″E
C_2	Centre	Värnamo, SE	57°10′59.38″N	14°02′52.15″E
C_1	Centre	Mårum, DK	56°01′35.80″N	12°16′51.78″E
S_4	South	Meldorf, GE	54°05′23.93″N	9°04′31.76″E
S_3	South	Ostbevern, GE	52°02′11.44″N	7°50′32.57″E
S_2	South	Heteren, NL	51°56′56.18″N	5°45′03.24″E
S_1	South	Hosingen, LU	50°03′11.34″N	6°04′40.66″E

For each of the 10 areas, 16 plants were propagated in the greenhouse. DNA extracted from fresh leave tissue was used to analyse the genetic variation with amplified fragment length polymorphism (AFLP) and the epigenetic variation with methylation-sensitive AFLP (MS-AFLP). Two samples failed to give reliable AFLP fragments (in C1 and S3) resulting in 158 samples and 160 samples for MS-AFLPs.

nutrient solution once a week. All plants were confirmed to be triploid, and thus apomictic, using a flow cytometer (Partec Ploidy Analyser) by checking their nuclear DNA content against a diploid reference plant (Tas & Van Dijk 1999).

DNA isolation, AFLP and MS-AFLP

DNA was isolated from approximately 1 cm^2 of leaf tissue following the CTAB procedure by Rogstad (1992) with minor modifications (Vijverberg *et al.* 2004). The leaf tissue was collected in microtubes and kept on ice, which contained two 1/8″ steel balls. Afterwards, the leaf tissue was homogenized in the CTAB buffer using a Tissuelyser II (Qiagen, the Netherlands). The DNA pellets were dissolved in 50 μL TE and stored at −20 °C until usage.

While the AFLP protocol uses the enzyme *Mse*I as the frequent cutter (Vos *et al.* 1995), the MS-AFLP protocol uses the DNA methylation-sensitive enzymes *Msp*I and *Hpa*II in parallel batches (Xiong *et al.* 1999; Keyte *et al.* 2006), each in combination with the same rare cutter *Eco*RI (Reyna-Lopez *et al.* 1997). *Msp*I and *Hpa*II are isoschizomers that recognize the same tetranucleotide sequence, 5′-CCGG, whereas the cytosines can be methylated on one or both DNA strands, referred as hemi and fully methylated. *Msp*I and *Hpa*II cut depending on the exact methylation status of the restriction site (e.g. see Schulz *et al.* 2013): both enzymes cut if the restriction site is free from cytosine methylations (type I), only *Msp*I cuts if the internal cytosine is hemi- or fully methylated (type II), only *Hpa*II cuts if the external cytosine is hemimethylated (type III), and additionally, sites that are fully methylated at the external cytosine or hemi- or fully methylated at both internal and external cytosines are not accessible for *Hpa*II and *Msp*I (type IV). The advantage of screening with both isoschizomers is the possibility to distinguish DNA methylation polymorphism from genetic polymorphism, where the fragment is absent due to mutation at the restriction site (Schulz *et al.* 2013).

The protocol for AFLP and MS-AFLP was adapted from Keyte *et al.* (2006) with some modifications. Based on previous pilot tests, we selected four *Eco*RI/*Mse*I primer combinations for AFLP analysis (AAC/CTA, AAC/CAA, AAC/CTT and ACC/CTA) and seven *Eco*RI/*Msp*I-*Hpa*II primer combinations for the MS-AFLP analysis (ACA/TAC, ACA/TCA, AAC/TAG, AG/TCA, AG/TAC, ACC/TCA and ACC/TAG). In Table S1 (Supporting information), all adapters and primers used for the AFLP and MS-AFLP protocol are summarized. Fifty nanograms of DNA was digested for three hours at 37 °C in a total volume of 20 μL with ten units of *Eco*RI (100 000 U/mL), *Mse*I (50 000 U/mL), *Msp*I (100 000 U/mL) or *Hpa*II (50 000 U/mL). The corresponding buffer was added to the digestion mix, and on top of that for the digestion with *Mse*I, we added 2 μg of BSA (restriction enzymes, restriction buffer and BSA; New England BioLabs, Bioke, the Netherlands). Adapters were then ligated in a total reaction volume of 30 μL containing: 1 unit of T4 DNA ligase and corresponding ligase buffer (ThermoFisher scientific, the Netherlands), 3.75 pmol of *Eco*RI adapter and, respectively, 37.5 pmol of *Mse*I or *Msp*I/*Hpa*II adapter for 18 h at 22 °C followed by 10 min at 65 °C. The ligation product was diluted to 15% in sterile water. Pre-amplification was performed in a total volume of 50 μL using 1 × buffer, 125 nmol MgCl2, 2.5 U Taq DNA polymerase (all from GC biotech BV, the Netherlands), 10 nmol dNTPs (ThermoFisher scientific), 15 pmol of each preselective primer (Table S1, Supporting information) and 10 μL of diluted ligation product. The reaction started with 2 min hold at 72 °C followed by 20 cycles of 30 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C and finished with 10-min incubation at 60 °C and hold at 10 °C. These pre-amplified products were diluted to 5% in sterile water. The selective amplification was performed in a total volume of 25 μL containing 1 × buffer, 37.5 nmol MgCl2, 1.25 U Taq DNA polymerase (all from GC biotech B.V.), 7.5 nmol dNTPs (ThermoFisher scientific), 10 μg BSA, 5 pmol labelled selective *Eco*RI primer, 20 pmol selective *Mse*I, *Hpa*II/*Msp*I primer and 5 μL diluted PCR product. The selective amplification was started with 2-min hold at 94 °C, followed by 10 cycles of 30 s at 94 °C, 30 s at 65 °C, 2 min at 72 °C and 25 cycles with 30 sec at 94 °C, 30 s at 56 °C, 2 min at 72 °C and ended with 10 min at

60 °C before hold at 10 °C. The final PCR product was diluted to 2.5% in sterile water and analysed on the ABI 3130 genetic analyser (Life Technologies Europe BV, the Netherlands).

To avoid systematic biases, we used a randomized block design to run all samples through the MS-AFLP and AFLP protocols. The samples were divided into four blocks, and each block was divided into four sub-blocks, each sub-block containing one individual per population. Additionally, 10% of the total number of samples was run as technical duplicates in order to quantify error rates and 10% as negative controls to check for peaks that indicate contamination signals and carry over effects (Bonin *et al.* 2007). Samples were fully randomized within sub-blocks, and blocks went through the laboratory protocols sequentially. This procedure ensured that any block-specific technical biases are randomly distributed over the ten populations and do not cause a specific bias that is correlated with the transect.

Fragment scoring

The fragments were analysed and scored using GENEMAPPER 3.7 (Life technologies Europe BV). Fragments between 100 and 500 base pairs were scored, and fragments that showed up in any of the negative controls were discarded. We used a semi-automated bin setting to identify marker loci as bins that had at least one sample showing a peak height above 50. Markers were scored as 'present' when peak height exceeded a relative peak-specific threshold (mean peak height minus two times the standard deviation) and if peak height exceeded a minimum absolute threshold of 10. Monomorphic loci, singletons and doubletons (i.e. when only 1–2 samples had a deviating status) were discarded. In a preliminary data analysis, we detected a significant block-specific bias: a subset of fragments was present in nearly all samples from one block but never in samples from any of the other blocks. We subsequently tested each marker for association with blocks using logistic regression, and we excluded all markers from further analysis that showed a significant block effect ($P < 0.05$). Additionally, we discarded all loci from analysis that showed more than two mismatches across the 16 pairs of duplicates. This resulted in a final data set of 85 polymorphic AFLP loci in 158 samples and 96 polymorphic MS-AFLP loci in 160 samples (Table 1).

The profiles for the selected *Msp*I and *Hpa*II markers were combined into a matrix of the four possible methylation conditions: type I) fragment is present in both *Msp*I and *Hpa*II profiles, type II) fragment is present only in *Msp*I profile, type III) fragment is present only in *Hpa*II profile and type IV) the absence of fragment from both profiles. Type II is often interpreted as evidence for CG methylation and type III is often interpreted as CHG methylation (Schulz *et al.* 2013), but this interpretation is questioned (Fulneček & Kovařík 2014). Type IV can have multiple causes: both inner and outer cytosines are hemi- or fully methylated, the outer cytosine is fully methylated, and a true fragment absence due to a sequence polymorphism in the restriction site condition (Salmon *et al.* 2008). Due to its uninformative status, we excluded fragments of type IV from logistic regression analysis. For multivariate analyses of cytosine methylations, we followed the analysis approach of Schulz *et al.* (2013) and we recoded the MS-AFLP combined matrix into two data sets: data set M containing methylated loci where the methylated state (types II and III) equals 1 and the unmethylated state (type I) equals 0, and data set U representing unmethylated loci where type I is scored as 1 and types II and III are scored as 0. Both matrices M and U contain the same information, but downstream analysis based on pairwise distance matrix that emphasize shared 1′s can differ between the M and U coding. As pointed out by Schulz *et al.* (2013), functionally different patterns may emerge when emphasizing shared methylated sites or shared unmethylated sites in the genome. In both M and U matrices, ambiguous type IV loci were coded as zeros following Schulz *et al.* (2013). Scoring error rates based on the 16 replicate samples were 4.3%, for the AFLP profile, 6.5% for the *Msp*I profile and 5.0% for *Hpa*II. Because we used a randomized design for the greenhouse experiment and for the laboratory protocols, the scoring errors are randomly distributed over the experimental design and therefore may cause undesired noise but no systematic bias in the results. Additionally, we evaluated how the patterns detected in our study are affected using different criteria of repeatability and error rates. Using lower error rates reduced the number of loci retained in the analyses considerably, leading to undesirably small data sets. However, we found that results remained qualitatively well comparable (Tables S2–S7, Supporting information).

Statistical analysis

To check for broad geographic patterns in Europe, we partitioned the transect into three regions: south, centre and north (Figs 1 and 3) and we performed several analyses to detect differentiation and diversity patterns at the regional level. In addition, more fine-grained patterns were analysed at the levels of population or/and latitude. First, clonal lineages were identified using GENOTYPE based on the AFLP profiles. Assuming

some level of scoring error and within-lineage mutation, this program uses the empirical distribution of all pairwise genetic distances between samples to set an appropriate threshold for lineage assignment; this distribution is typically bimodal as a result of within-lineage variation and genetic variation between lineages and the appropriate threshold lies in between (Meirmans & Van Tienderen 2004). Pairwise distances between individuals were based on dice similarities: 1−(2a/(2a+b)), where a is the number of shared 1's and b the number of the number of loci with discordant information. In our data, ten mismatches of a total of 85 polymorphic loci were allowed as a maximum distance between lineage members (Fig. S1, Supporting information). Clonal diversity within populations and within regions was captured as the number of clonal lineages divided by total number of plants per group. Shannon–Weaver indices were calculated for small sample sizes as an additional measure of clonal diversity (Chao & Shen 2003) using GENODIVE (Meirmans & Van Tienderen 2004). The regional differences within these Shannon–Weaver indices were then tested using a bootstrapping approach, that is resampling the individuals from the regions and comparing the indices (Manly 1991).

Second, multivariate analyses were performed that detect genomic diversity by quantitatively analysing the calculated pairwise dice similarity scores. PCoAs were plotted for AFLP and MS-AFLP (M&U) profiles based on the first two dimension calculated with the R-function pcoa() from the package Ape with an additive constant to modify the nondiagonal distances to Euclidean (Cailliez 1983) and hence can be represented in n − 1 dimensions. A hierarchical AMOVA (amova(), R-function from the package Pegas with 10 000 permutations) was performed to evaluate genetic (AFLP loci) and epigenetic variation (MS-AFLP loci from M and U profiles) among regions, among populations within regions and within populations. Fst was calculated and averaged across all loci for the AFLP and M profiles (fst(), R-function from package Vegan). Permutation tests for homogeneity of multivariate dispersion were calculated with 999 permutations (betadisper() and permutes(), R-functions from package Vegan). This is a multivariate analogue to Levene's homogeneity of variances test; it evaluates whether different groups have different levels of variation, irrespective of differences in group means.

Correlations between AFLP, MS-AFLP and geographic distances were tested using mantel tests (mantel() with 999 permutations from the R package Vegan). Geographic distances were either coded as km distances between the 10 populations, or as proxies for regional distances: same region = 0, adjacent regions = 1 and nonadjacent regions = 2. Of special interest is the partial mantel correlation test (partialmantel() with 999 permutations from Vegan) between MS-AFLP loci and geographic or regional distances, after controlling for the effect of genetic distances. This captures epigenetic differentiation that is uncorrelated with genetic differentiation.

In addition to the pairwise distances based multivariate analyses that describe genomewide patterns of variation, we analysed single markers individually. Logistic regression models evaluate whether the marker status of the M profile of MS-AFLP and the AFLP profile associates with region, population and the latitude of the sampling site. As mentioned above, we handled here the ambiguous type IV status as missing data for the M profile. This analysis was performed with the R-function *glm*() using binomial error distribution and a logit link function. The *P*-values where corrected for multiple testing at a false discovery rate of 0.05 with the function *p. adjust*().

Results

Clonal diversity

The AFLP profile consisting of 158 samples revealed 63 clonal lineages (with a maximum of 10 marker differences allowed within lineage, see Fig. S1 supporting information). The 15–16 sampled plants per population represented on average 9–13 different clonal lineages. The regional clonal diversity showed a weak decrease from south (52%) to centre (51%) to north (48%). This decrease in diversity was supported by a decrease in the corrected Shannon–Weaver index: south (1.6), centre (1.5) and north (1.4) with a significant clinal pattern along the transect: south > centre ($P = 0.05$) and south > north ($P < 0.01$; *P*-values based on bootstrapped indices with 9999 permutations). Most clonal lineages occurred exclusively in a single region. Thirteen clonal lineages occurred in multiple regions: four widespread lineages were found in all three regions, centre and north shared four lineages, centre and south shared three lineages, and north and south shared two lineages (Fig. 2, Table S8, Supporting information).

Genetic and epigenetic variation

AMOVA revealed that the great majority of the molecular variation (92% of the genetic variance, and 96% of the epigenetic M and 97% of the epigenetic U profiles) was partitioned within populations, while the small remaining portion was partitioned among populations within regions and among regions (Table 2). Lack of strong regional differentiation is also visible in the principal coordinate analysis (PCoA) plots that are

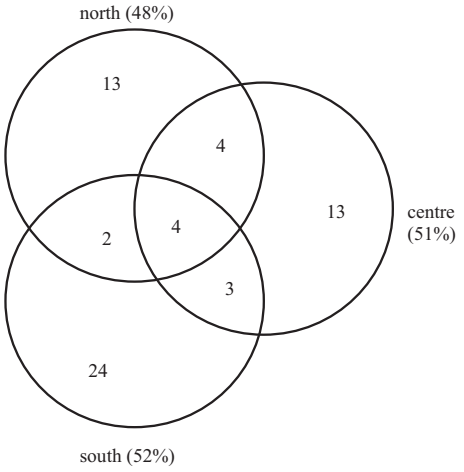

Fig. 2 Venn diagram with number of clonal lineages per region. In total, 63 clonal lineages were found based on the amplified fragment length polymorphism (AFLP) data set. In brackets, the percentage of clonal diversity within region is shown: number of clonal lineages divided by total number of plants per region.

Table 2 Variance partitioning (AMOVA) for amplified fragment length polymorphisms (AFLP) and methylated/unmethylated profiles based on methylation-sensitive AFLPs (MS-AFLP)

	Among regions	Among populations within regions	Within population
AFLP			
Df	2	7	148
SSD	0.174	0.223	2.988
Mol. var. (%)	4.8	3.4	91.8
P	<0.01	ns	
MS-AFLP methylated			
Df	2	7	150
SSD	0.771	1.286	19.936
Mol. var. (%)	2.1	2.0	95.9
P	<0.01	ns	
MS-AFLP unmethylated			
Df	2	7	150
SSD	0.164	0.540	7.693
Mol. var. (%)	0.2	3.1	96.7
P	ns	<0.01	

Table shows the output of R-function amova() from package Pegas. Df: degrees of freedom. SSD: sum of square deviation. Mol. var (%): Molecular variation percentages derive from variance components sigma 2. P: *P*-values deriving from 10 000 permutations, ns: not significant.

based on pairwise AFLP and MS-AFLP distances (Fig. 3). Despite the small percentage of variation partitioned among regions, these variance components were significant for the genetic and methylated variation profiles (Table 2). The regional differentiation was somewhat more pronounced in the AFLP than in the methylated data: genetic regional differentiation of 4.8% compared to methylated regional differentiation of 2.1%. Analysis of single markers also showed stronger genetic than epigenetic differentiation, with only few MS-AFLP markers significantly associated with regions (Table 3). Consistent with the limited regional differentiation, measures of genetic subdivision among populations showed low values: $Fst_{AFLP} = 0.04$ and $Fst_{MS\text{-}AFLP} = 0.027$, indicating high migration across the populations.

Within regions, levels of MS-AFLP variation were higher than levels of AFLP variation, especially in the M profiles (Fig. 4). The analysis of within-region genomic diversity, that is average distance to the regions' centroid, did not show a clinal pattern along the south-to-north transect (permutation test: $P > 0.05$; Fig. 4).

Genetic and epigenetic correlation

Because the regions north, centre and south are distributed along a linear transect, differentiation between these regions may derive from adaptation to the regional conditions or from neutral isolation by distance. Correlations between genetic and geographic distances (km distances between populations) were weak yet significant ($R = 0.106$, $P = 0.001$), and epigenetic variation showed an even weaker correlation with geographic distance (using the epigenetic M profile: $R = 0.048$, $P < 0.05$ but not significant for U profiles). Additionally, we used partial mantel tests to detect geographic patterns of epigenetic variation after controlling for genetic effects; that is, we looked for geographic patterns in the MS-AFLP data that did not simply mirror geographic patterns in the genetic data. When testing the correlation between autonomous MS-AFLP profiles and geographic km distances no evidence was found at the level of populations. When tested at the regional level, after correcting for AFLP variation, a significant correlation was observed between MS-AFLP profiles and regional distances (M profile: partial mantel correlation $R = 0.049$, $P < 0.05$; not significant for the U profile MS-AFLP data).

Visualization of the four most common clonal lineages (occurring in all three regions, see Table S8, Supporting information) shows genetic clustering but limited clustering based on their epigenetic profiles (Fig. 5). While there is a clear overall correlation between AFLP and MS-AFLP profiles across all clonal lineages (Mantel test correlation between AFLP and M profiles: $R = 0.163$, $P = 0.001$; AFLP and U profiles: $R = 0.068$, $P = 0.07$), this absence of obvious epigenetic clustering supports the idea that there is also some

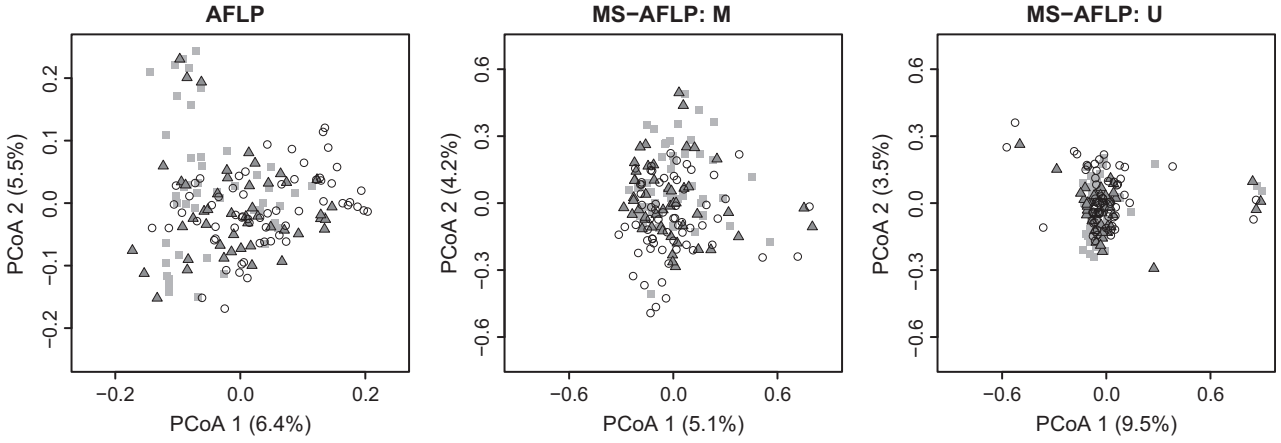

Fig. 3 Principal coordinate analyses based on genetic (AFLP) and epigenetic (MS-AFLP) distances shown as methylated loci (M) and unmethylated loci (U) based on the mixed scoring approach (see Materials and Methods). Regions are displayed by colour: white circle = south, black triangle = centre, grey rectangle = north.

Table 3 Results for single-marker tests using generalized linear models. Number of significant loci (*P*-value adjusted for multiple testing at FDR 0.05) and proportion of differentiated genetic and epigenetic loci that associate with population, region or latitude

	Significantly differentiated	
	AFLPs (n = 85)	MS-AFLPs (n = 96)
Differentiation by		
Population	15 loci – 18%	ns
Region	12 loci – 14%	2 loci – 2.1%
Latitude	5 loci – 6%	ns

n = total number of polymorphic loci.

fraction of the DNA methylation variation that is independent from genetic background.

Discussion

In this study, we explored patterns of epigenetic variation in apomictic dandelion populations along a northward range expansion gradient. We hypothesized that range expansion could result in certain patterns of epigenetic variation: (i) increased levels of epigenetic variation towards the north and (ii) in regional epigenetic differentiation. We found limited evidence for regional sequence-independent epigenetic differentiation and no gradient in levels of epigenetic variation. While much of the heritable epigenetic variation was intertwined with genetic variation, a fraction of the DNA methylation differentiation between regions along the transect was not associated with genetic variation. This autonomous fraction of epigenetic variation is interesting because it shows a potential contribution to phenotypic variation and plasticity beyond what can be explained by genetic variation; however, this fraction is quite small.

Genetic and epigenetic patterns in apomictic dandelions

The analysis of the offspring of sampled apomictic dandelions along the latitudinal transects revealed very high clonal diversity. Novel apomictic lineages are continuously formed from mixed sexual-apomictic populations and subsequently migrate northwards. That probably accounts for the high clonal diversity observed in all sampling locations, which is consistent with previous reports (Van der Hulst *et al.* 2001). We observed a moderate decrease in clonal diversity towards the north, possibly reflecting clonal selection in response to environmental variability when migrating away from the location of origin.

The hypothesis that epigenetic variation increases towards the north was not supported. This may be because exposure to novel climatic conditions does not trigger enhanced levels of epigenetic variation. An alternative explanation is that such an epigenetic signal is very transient and is not reflected anymore in present-day standing variation. Also, increased biotic stress exposure towards the north might be partly counteracted by reduced abiotic stress levels along the same gradient (Verhoeven & Biere 2013), resulting in similar overall levels of stress along the transect. Modest levels of differentiation in epigenetic variation were observed at a regional, local and clinal level along the transect as hypothesized, which suggests environment-related epigenetic patterns. Such environment-associated epigenetic differentiation could arise from either induction of heritable epigenetic modifications by the environment or divergent selection on stable epimutations.

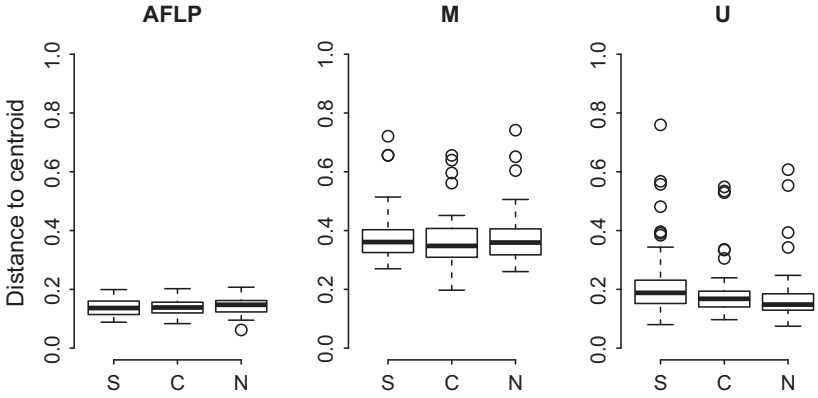

Fig. 4 Within-region genomic diversity. Box plots represent the average distance to centroid calculated at the regional level. Data for S (south), C (centre) and N (north) regions, and for AFLP, M (methylated) and U (unmethylated) profiles.

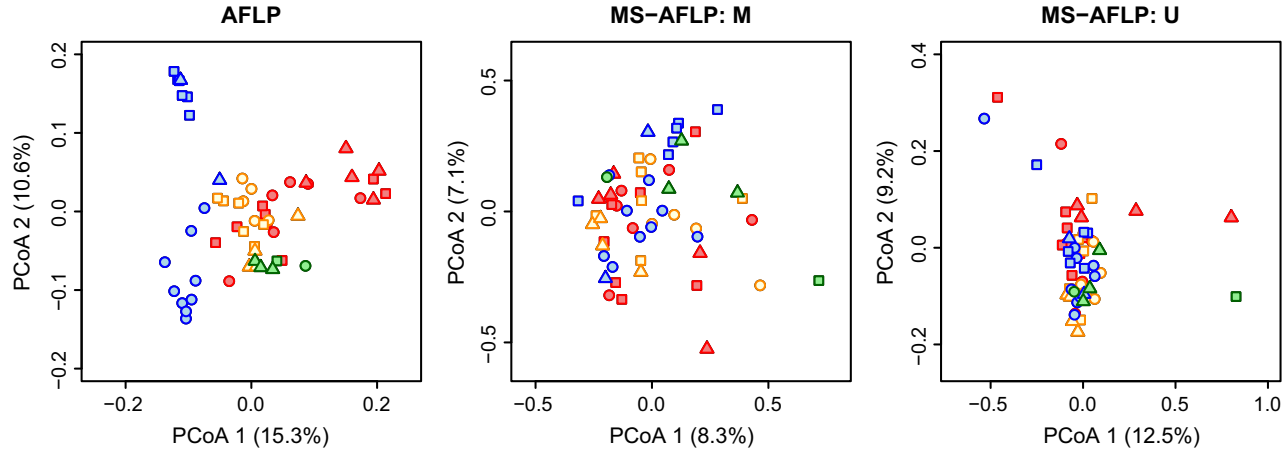

Fig. 5 The four most common clonal lineages are displayed by colour in the principal coordinate analyses of genetic (AFLP) and epigenetic (MS-AFLP) distances shown as methylated loci (M) and unmethylated loci (U). Symbols indicate the regions: circle = south, triangle = centre, rectangle = north.

Autonomous epigenetic variation

The correlation between genetic and epigenetic variation observed in our study shows that a large part of the epigenetic variation is not sequence independent which may imply that much of the epigenetic variation is not autonomous but rather under genetic control (Richards 2006). To date, epigenetic polymorphism has generally shown association with DNA sequence, for example with transposable elements (Paszkowski & Grossniklaus 2011; Schmitz *et al.* 2013). It is important to point out that the observed correlation can either derive from genetic control over DNA methylation patterns, or from the build-up of lineage-specific (and potentially autonomous) epimutations within clonal lineages that may also create statistical associations between genetic and epigenetic patterns. It has been shown for *Arabidopsis thaliana* that differences in DNA methylation status can accumulate over generations similar to, but less stable than genetic mutations (Becker *et al.* 2011). In our study, in addition to sequence-associated DNA methylation, small but significant regional epigenetic differentiation persisted after controlling for the correlation with genetic variation. This portion of epigenetic variation likely reflects epigenetic differentiation that is not under genetic control, and such autonomous, heritable epigenetic differentiation may contribute to phenotypic variation that cannot be explained by genetic variation alone. It has been proposed that such additional epigenetically mediated phenotypic variation could play a role in plant adaptation to rapidly changing conditions (Bossdorf *et al.* 2008; Massicotte & Angers 2011). We detected this autonomous fraction of epigenetic variation only in the M profiles (which emphasizes shared cytosine methylation between plants) and not in the U profiles (which emphasizes cytosine that are not methylated). In the interpretation of Schulz *et al.* (2013), this could indicate a larger contribution to differentiation of epigenetically silenced loci compared to transcriptionally active loci.

Natural epigenetic variation

Several recent studies have revealed an association of natural epigenetic variation with environment-specific traits within genetically uniform groups (Gao *et al.* 2010; Lira-Medeiros *et al.* 2010; Richards *et al.* 2012). Studies in natural populations of sexually reproducing plants demonstrated a correlation between genetic and epigenetic markers, while a proportion of epigenetic variation showed sequence-independent differentiation (Li *et al.* 2008; Herrera & Bazaga 2010; Schulz *et al.* 2014). Also, in natural populations of animal species and in nectar-inhabiting yeast, some evidence for distinct and ecologically relevant epigenetic patterns was found (Herrera *et al.* 2011; Massicotte *et al.* 2011; Schrey *et al.* 2012). Our study differs from these and related studies because we did not base directly on field-collected material (or vegetatively derived offspring), but on natural DNA methylation variation that persists through apomictic seed production. Hence, our findings contribute to the field of ecological epigenetics in natural populations by pointing out epigenetic differentiation in the component of natural DNA methylome variation that is heritable, which arguably is the most relevant fraction of epigenetic variation for adaptation (Bossdorf *et al.* 2008).

The detection of linear patterns of genetic variation and the presence of widespread clonal lineages along the transect are in line with the postglacial latitudinal range expansion of *Taraxacum officinale*. However, alternative historical migration routes may exist as well; apomictic dandelions also persisted in glacial refugia in south-eastern Europe and may have colonized Sweden entering from the north and migrating to the south. In support of this possibility, we observed a distinct group of a few samples in the AFLP data set (upper left corner in Fig. 3A) that might reflect plants originating from a different glacial refugium. However, these plants did not have deviating MS-AFLP profiles (Fig. 3B and C). If these individuals would indeed represent a group of plants with a different historical background, the similarity of their epigenetic profiles would further support our main conclusion that regional epigenetic differentiation exists partly independent of the genetic background.

Detecting cytosine methylations

To compare genetic and epigenetic variation, we ascertained the detection of purely epigenetic variation by scoring a combination of the two methylation-sensitive enzyme profiles (*Msp*I and *Hpa*II). The ability to detect purely epigenetic variation (autonomous from genetic variation) is an important strength of MS-AFLPs, and this method has been used successfully to describe patterns of epigenetic variation in a wide range of different species (e.g. Cervera *et al.* 2002; Salmon *et al.* 2008; Herrera *et al.* 2011; Massicotte *et al.* 2011). However, there are also a number of technical limitations of MS-AFLPs, including the relatively low numbers of loci and the lack of information about sequence context (Becker *et al.* 2011; Schrey *et al.* 2013). Better methylome screening is possible, for example whole-genome bisulphite sequencing (Becker *et al.* 2011) or reduced representation bisulphite sequencing (Meissner *et al.* 2005). However, these and other sequencing-based methods are not yet cost-effective when using sample sizes typical for ecological population studies and are challenging to use in species without a reference genome.

Conclusion

Natural populations of apomictic dandelions along a northward range expansion gradient revealed high levels of heritable genetic and epigenetic variation, but limited regionally structured variation and no enhanced epigenetic variation with increasing latitude. Therefore, we did not find evidence of increased levels of inherited DNA methylations in northern, potentially more stressful, environments. The observed regional differentiation is partly correlated with genetics and partly non-correlated. In addition to within-lineage genetic variation, it is this sequence-independent epigenetic variation that may contribute to phenotypic variation and adaptation in asexual plant lineages. Studies like ours can demonstrate the potential of epigenetic variation in natural populations, but to understand its functional consequences, studies that link DNA methylations to their function and detect epigenetic variation at higher resolution are necessary.

Acknowledgements

We thank Kitty Vijverberg and Niels Wagemaker for advice on the DNA extraction method and for support on the MS-AFLP protocol. Thanks to Thomas van Gurp, Maria Johansson and Farag Mahmoud El Sabbagh for their help during the seed collection trip and to Tanja Bakx-Schotman and Slavica Milanovic-Ivanovic for their help in collecting leaf material for DNA extraction. Thanks to Patrick Meirmans for support on using the program GENOTYPE and to Christina Richards for useful comments on the manuscript. This work was supported by NWO-ALW grants 884.10.003 and 864.10.008 from the Netherlands Organisation for Scientific Research. This is publication 5905 of the Netherlands Institute of Ecology (NIOO-KNAW).

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V.P. performed the research and data analysis and contributed to study design. B.S. contributed to data analysis. C.O. contributed to molecular laboratory work and manuscript writing. A.B. and W.P. contributed to the study design and manuscript writing. K.V. designed the study and contributed to data analysis and manuscript writing.

Data accessibility

AFLP profile and *MspI/HpaII* combined MS-AFLP profile plus error rate calculations and the genetic and geographic distances (input files for AMOVA, multivariate dispersion test, Mantel tests and PCoAs): DRYAD, doi:10.5061/dryad.f7g6r.

Supporting information

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

Table S1 Adapters and primers used for AFLPs and MS-AFLPs.

Table S2 Scoring error per error criteria.

Table S3 Significantly differentiated loci associated with population, region and latitude.

Table S4 Variance Partitioning (AMOVA) for amplified fragment length polymorphisms (AFLP) and methylated/unmethylated profiles based on methylations sensitive AFLPs (MS-AFLP).

Table S5 Analysis of homogeneity of multivariate dispersion: *P*-values from permutation tests on the distances to group centroids.

Table S6 Mantel tests between genetic and epigenetic profile.

Table S7 Mantel tests and Partial Mantel test between genetic/epigenetic profile and geographic distances.

Table S8 Clonal lineage assignment.

Fig. S1 Selecting maximum genetic distance between lineage members.