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Manipulation of cytosine methylation does not remove latitudinal clines in two invasive goldenrod species in Central Europe

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Abstract

Invasive species frequently differentiate phenotypically in novel environments within a few generations, often even with limited genetic variation. For the invasive plants *Solidago canadensis* and *S. gigantea*, we tested whether such differentiation might have occurred through heritable epigenetic changes in cytosine methylation. In a 2-year common-garden experiment, we grew plants from seeds collected along a latitudinal gradient in their non-native Central European range to test for trait differentiation and whether differentiation disappeared when seeds were treated with the demethylation agent zebularine. Microsatellite markers revealed no population structure along the latitudinal gradient in *S. canadensis*, but three genetic clusters in *S. gigantea*. *Solidago canadensis* showed latitudinal clines in flowering phenology and growth. In *S. gigantea*, the number of clonal offspring decreased with latitude. Although zebularine had a significant effect on early growth, probably through effects on cytosine methylation, latitudinal clines remained (or even got stronger) in plants raised from seeds treated with zebularine. Thus, our experiment provides no evidence that epigenetic mechanisms by selective cytosine methylation contribute to the observed phenotypic differentiation in invasive goldenrods in Central Europe.

KEYWORDS

common-garden experiment, epigenetic variation, microsatellites, *Solidago canadensis*, *Solidago gigantea*, zebularine

1 | INTRODUCTION

The collapse of biogeographical barriers during the Anthropocene (Lewis & Maslin, 2015; Steffen et al., 2011) has resulted in >13,000 plant species that have become naturalized outside their native range (Pyšek et al., 2017; van Kleunen, Dawson et al., 2015). Some of these have become successful invaders (Turbelin et al., 2017). This is remarkable because introduced species should be less adapted to their new environments than native species, and are thought to have limited adaptive potential due to genetic bottleneck events during introduction (Pérez et al., 2006). Nevertheless, several invasive species show signatures of rapid post-introduction evolution (Colautti & Barrett, 2013; Colautti & Lau, 2015;

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Joshi & Vrieling, 2005; Oduor et al., 2016; Pal et al., 2020; Pérez et al., 2006; Zhang et al., 2018), for example latitudinal clines in flowering phenology (Alexander et al., 2012; Santamaría et al., 2003; Weber & Schmid, 1998). However, little remains known about the mechanisms that allowed alien species to rapidly generate phenotypic differentiation along environmental gradients.

Heritable epigenetic variation has recently been proposed as a putative driver of adaptation to novel environments (Bonduriansky & Day, 2009; Bossdorf et al., 2008; Prentis et al., 2008), indicating a possible role in the rapid adaptation of invasive plants (Banerjee et al., 2019; Hawes et al., 2018; Prentis et al., 2008; Smithson et al., 2019). Epigenetic variation refers to phenotypic variation that is not caused by changes in nucleotide sequences, but changes in gene expression, for example due to differences in histone packaging (Liu et al., 2010) or cytosine methylation (Bewick & Schmitz, 2017; Niederhuth & Schmitz, 2017). The latter has to date been the most thoroughly studied epigenetic mechanism inducing variation in gene expression at several levels that may indirectly affect gene regulation (Henderson & Jacobsen, 2007). Examples of such methylation-based regulatory mechanisms include reversible marking of boundaries between euchromatin and heterochromatin at ^{m}CHH (H = A, C, T) sites mediated by small interfering RNA (siRNA) (Khraiwesh et al., 2012; Li et al., 2015; Roudier et al., 2009), changes in the expression state of epialleles mediated by repeat sequences near the affected gene (Weigel & Colot, 2012), or methyltransferase enzymes that transfer or de novo create methylation marks at CpG, CpHpH or CpNpG (N = A, C, G, T) sites (Cao & Jacobsen, 2002). These mechanisms play a role in phenotypic plasticity (Nicotra et al., 2010) and stress-related adaptation in plants (Boyko & Kovalchuk, 2008) and can be transmitted to the next generation (Henderson & Jacobsen, 2007; Jablonka & Raz, 2009; Thamban et al., 2019; Verhoeven & van Gurp, 2012). The involvement of epigenetic regulation in ecological and evolutionary responses could resolve the paradox of rapid local phenotypic differentiation in invasive plant species with limited genetic variation and a short time since introduction (Richards et al., 2012; Schrieber & Lachmuth, 2017). Although several papers support this idea (Baker et al., 2018; Cubas et al., 1999; Herman & Sultan, 2016; Kalisz & Purugganan, 2004; Richards et al., 2012; Vergeer et al., 2012), experimental studies that disentangle epigenetic and genetic contributions to phenotypic variation are still relatively rare.

Here, we tested for two invasive goldenrod species, *Solidago canadensis* sensu *lato* (*s.l.*) L. and *S. gigantea* Aiton (Asteraceae), whether they show heritable phenotypic latitudinal clines in fitness-related traits in their invasive range in Europe, and whether this variation is affected by cytosine demethylation. We also used microsatellite markers to analyse population structure in both species. *S. canadensis* and *S. gigantea* are native to North America, but were introduced to Europe in the 17th and 18th century, respectively (Aiton, 1813; Weber, 2017). Although their introduction dates back at least 250 years, both species show reduced genetic variation in their invaded European range, indicating a bottleneck or founder effect (Alexander, Poll et al., 2009; Schlaepfer et al., 2008). Weber and Schmid (1998) previously conducted a common-garden experiment with plants from invasive populations of *S. canadensis s.l.* (named *S. altissima* in their publication, but which is now considered the morphologically similar diploid *S. canadensis s.l.*; hexaploid plants of *S. altissima* have so far been found only in Belgium; Verloove et al., 2017) and *S. gigantea* raised from field-collected rhizome cuttings, and showed that with increasing latitude of origin, plants flowered earlier and at a smaller size. These clines in phenology and size could reflect vegetative carry-over effects (Dong et al., 2018). To test whether such clines are transmitted sexually, we collected seeds of 25 *S. canadensis* and 24 *S. gigantea* populations along a latitudinal gradient from Switzerland to southern Sweden, and grew the offspring in a common garden in Konstanz, Germany. To test for the role of cytosine methylation on phenotypic expression, we treated half of the seeds per mother plant with the demethylation agent zebularine (Baubec et al., 2009).

Our study aimed to answer three main questions: (i) Do *S. canadensis* and *S. gigantea* show latitudinal clines in phenology and fitness-related traits when plants are grown from seeds (and not from rhizomes as in Weber & Schmid, 1998)? (ii) Does treatment with a demethylation agent affect phenology and fitness-related traits, and putative latitudinal clines therein? Specifically, do clines become less pronounced after treatment of seeds with zebularine? (iii) Do *S. canadensis* and *S. gigantea* show neutral molecular genetic variation, and if so, is this variation structured along the analysed latitudinal gradient?

2 | MATERIALS AND METHODS

2.1 | Study species and seed collection

Solidago canadensis and *S. gigantea* are native to North America, but were introduced to Europe in the 17th and 18th century, respectively (Aiton, 1813; Weber, 2017). Both species are perennial herbaceous plants that can grow over 2 m in height, spread vegetatively via rhizomes (Klimešová et al., 2017), and usually flower in late summer and early autumn with branched inflorescences that can produce >10,000 seeds (Weber, 2000). The above-ground parts die back in winter, and plants resprout, usually with multiple ramets, from their rhizomes (Egli & Schmid, 2000; Weber & Jakobs, 2005). Both species have become highly invasive in ruderal and disturbed areas (*S. canadensis*; van Kleunen & Schmid, 2003), and in mesic habitats such as wetlands (*S. gigantea*; Weber & Jakobs, 2005). Whereas in their native range both species have multiple ploidy levels (Semple & Cook, 2006), in Europe *S. canadensis s.l.* is diploid (van Kleunen & Schmid, 2003; but see Verloove et al., 2017, for a recent single record of hexaploid *S. altissima* in Belgium) and *S. gigantea* is tetraploid (Schlaepfer et al., 2008, 2010). We collected ripe seeds from 25 populations of *S. canadensis* and 24 populations of *S. gigantea* along a latitudinal gradient in Central Europe between October 2014 and March 2015 (Figure 1). For each population and species, we collected seeds from at least five mother plants that were at least 1 m

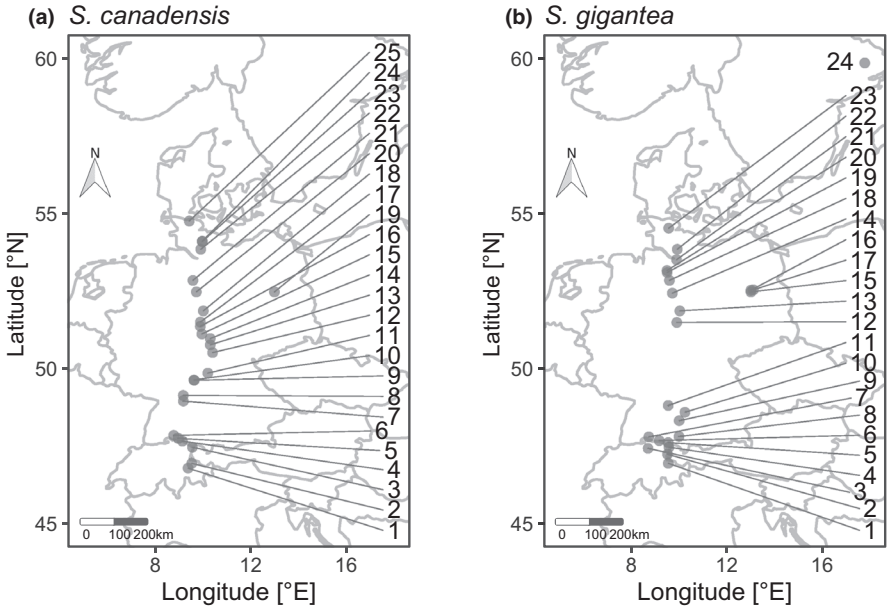

FIGURE 1 Sampling locations along the analysed latitudinal gradient in Central Europe. Populations of (a) *Solidago canadensis* and (b) *S. gigantea* are numbered according to their positions along the gradient (see Tables S1 and S2 for additional information)

apart to minimize sampling from the same clones. Information on sampling location, elevation (m a.s.l.), estimated numbers of shoots per population and the number of maternal lines are given in Tables S1 and S2.

2.2 | Zebularine demethylation treatment

Zebularine is a cytidine analogue ($C_9H_{12}N_2O_5$) that is commonly used as a demethylation agent (Alonso et al., 2017; Baubec et al., 2009; Herman & Sultan, 2016; Verhoeven & van Gurp, 2012). By inhibiting DNA methyltransferases, zebularine changes cellular methylation patterns on cytosine locations and causes hypomethylation during mitosis (Baubec et al., 2009; Griffin et al., 2016). For example, treatment of *Arabidopsis thaliana* seeds with 80 µm zebularine during germination decreased methylation levels of plants from 81.4% to 58.8% and, in *Medicago sativa* seedlings, treatment with 40 µm zebularine decreased methylation levels from 20.6% to 17.6% on average (Baubec et al., 2009). Additionally, it reduced methylation levels at CpG sites from 17.8% to 15.9% in *A. thaliana* seedlings treated with 25 µm zebularine (Griffin et al., 2016). Although the effects are likely to be transient and restoring mechanisms are triggered already at the seedling stage (Liu et al., 2015), zebularine-derived hypomethylation has been reported to modify stress responses in different plant species (Baker et al., 2018; Verhoeven & van Gurp, 2012). However, treatment with zebularine was also reported to induce growth retardation and increased mortality of seedlings at concentrations > 50 µm for *Taraxacum officinale* (Verhoeven & van Gurp, 2012) and *Polygonum persicaria* (Herman & Sultan, 2016), and at concentrations >40 µm for *A. thaliana* (Baubec et al., 2009). Based on a pilot experiment using different concentrations of the demethylation agent zebularine (0, 12.5, 25, 50, 100 and 200 µm; see Method S1), we determined that goldenrod seedlings showed impaired growth at concentrations exceeding 25 µm when germinating on filter paper moistened with zebularine solution. We therefore chose to use the concentration at which growth disruptions were minimal (25 µm), but which was still likely to result in demethylation (see also Herman & Sultan, 2016).

For the main experiment, in mid-April 2015, seeds were surface-sterilized for 3 min in a 5% sodium hypochlorite (NaOCl) solution, rinsed in double-distilled water (ddH_2O) and dried with paper tissues. Two batches per maternal line, each containing 10–20 seeds, were placed on filter paper (Ø 2.7 cm, VWR) in separate Petri dishes (Ø 3.5 cm). Filter paper was moistened with either 200 µl ddH_2O (hereafter called control) or an aqueous solution of 25 µm zebularine (hereafter called zebularine-treated). Petri dishes were sealed (Parafilm, Bemis) and placed in randomized order in a growth chamber at the University of Konstanz (14-hr photoperiod, night–day cycle with temperature range 17.5–22.5°C and light intensity 110–135 µmol m^{-2} s^{-1}) and covered with a single layer of regular white paper to minimize condensation on the lids. Because of the potential instability of zebularine (Cho et al., 2011; Marquez et al., 2005), every second day, we transferred all zebularine-treated and control seeds to new Petri dishes with freshly prepared solutions until at least three seeds per dish had germinated (i.e., clearly visible cotyledons). This process was repeated until the beginning of May 2015 (*S. canadensis*) and end of April 2015 (*S. gigantea*).

2.3 | Plant precultivation

Two 3–5-day-old seedlings of each maternal line and treatment were transplanted into 7 × 7 × 6.5-cm pots (substrate: Pikiererde Classic, Einheitserdewerke Werkverband e.V.) and kept in a glasshouse until the end of May (*S. canadensis*) or mid-May (*S. gigantea*), when they were moved outside for acclimatization. One week later, plants were transplanted into circular 7-L pots (substrate as before with addition of 5 g/L Osmocote slow-release fertilizer; release: 12–14 months;

Everris International B.V). In total, we transplanted 400 *S. canadensis* plants (206 controls and 194 zebularine-treated plants, representing 25 populations and 224 maternal lines) and 336 *S. gigantea* plants (184 controls and 152 zebularine-treated plants, representing 24 populations and 189 maternal lines) to the common garden. Sample sizes per population and treatment are given in Tables S1 and S2.

2.4 | Experimental set-up of 2-year common-garden experiments

For each of the two species separately, we used a (incomplete) randomized block design, with eight blocks per species. We aimed to have each population represented in each block with one control and one zebularine-treated offspring of the same maternal line. For cases where control and zebularine-treated plants of the same maternal line were not available, we paired control and treated plants of different maternal lines from the same population. Blocks were arranged in two rows of four and each block consisted of 55 individuals in an 11 × 5 grid for *S. canadensis* and up to 46 individuals in an 11 × 4+2 grid for *S. gigantea*. Gaps due to an unequal number of surviving seedlings were filled with left-over seedlings of the same species as buffer plants. In addition, blocks were surrounded at each side by one row of buffer plants to reduce edge effects. Blocks were spaced at least 75 cm apart. The experimental area was exposed to full sunlight throughout the day and watered ad libitum to ensure moist substrate. A molluscicide (Schneckenkorn, Spiess-Urania Chemicals GmbH) was applied around pots and scattered on top of the soil surface inside pots at the beginning and middle of June in 2015 and at the end of April in 2016. In spring 2016, an additional 10 g of Osmocote slow-release fertilizer (release: 2–3 months; Everris International B.V.) was scattered on top of the soil surface inside pots.

2.5 | Plant measurements

At the beginning of the common-garden experiment in May 2015, we counted the number of true leaves (i.e., excluding cotyledons) and measured the length and width of the longest leaf per plant. We multiplied those values as an estimate of the initial leaf area per seedling (cm^2; hereafter initial size). The onset of flowering of each plant was monitored every other day, and defined as the time point of the first unfolded ray floret on any of the capitula. Additionally, stretched height (cm; hereafter called plant height) at flowering (when height growth ceases) was measured for each plant in both years. To test whether zebularine treatment during the seedling stage caused a sustained treatment effect reflected in plant growth at the beginning of the experiment, we analysed plant height measured on four occasions after transfer to the common garden (Method S2, Figures S1 and S2).

As a proxy for allocation to sexual reproduction, we harvested inflorescences from the tip down to the lowest side shoot once ripe seeds were observed (i.e., plants were harvested multiple times if they formed new flowers after harvesting; Figure S3). This was done to avoid the release of mature seeds and subsequent spread of the species in the garden and its surroundings. At the end of October 2015, when flowering had ceased, we harvested the remaining (vegetative) above-ground biomass to assess complete above-ground biomass as a fitness-related parameter and proxy for growth. Harvested biomass was dried at 70°C for >72 hr before weighing. After drying, we separated stem tissue from the inflorescence to get the reproductive biomass (g dry weight). Stems were then added to the vegetative biomass (i.e., any above-ground tissue not part of the reproductive biomass). The pots with roots and rhizomes were kept outside over winter. At the beginning of the second vegetation period (April 2016), we counted the number of ramets as a measure of vegetative reproduction.

2.6 | Molecular genetic variation

To analyse population genetic structuring, we genotyped one representative of each maternal line in the control treatment using 11 microsatellite markers (Table S3) that had been developed for *Solidago* spp. (Beck et al., 2014; Wieczorek & Geber, 2002). Two monomorphic markers (Sg1, Sg12) were excluded for both species and two further markers (Sg6, SS19D) for *S. canadensis* due to poor quality.

Two fully expanded, healthy leaves per plant were collected in August 2015 and silica-dried for DNA extraction (Chase & Hills, 1991) with a modified CTAB protocol (Doyle & Doyle, 1990) using 20 mg of dry leaf material per plant. DNA was amplified with the microsatellite markers and 5-FAM dye (Thermo Fisher Scientific) during polymerase chain reaction (PCR; 94°C for 4 min; three cycles of 94°C for 30 s; 63°C for 60 s; 72°C for 45 s; 35 cycles 94°C for 30 s; 61°C for 60 s; 72°C for 45 s; 72°C for 15 min) and samples were sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Peaks were scored manually using GENEMAPPER software version 5.0 (Applied Biosystems). This succeeded for 203 *S. canadensis* (25 populations) and 174 *S. gigantea* (24 populations) plants. We analysed microsatellite data in allelic format (see Tables S4 and S5 for summary statistics) and conducted a discriminant analysis of principal components (DAPC; Jombart et al., 2010) to assess population stratification of genotyped individuals per species using the package ADEGENET version 2.1.3.

DAPC does not assume any underlying population model or linkage disequilibrium and is a two-step procedure starting with dimension reduction of (scaled and centred) raw microsatellite data via principal components analysis (PCA). We retained the principal components (PCs) that accounted for 95% of the cumulative variance in the data for use in linear discriminant analysis that computes the contribution of each allele to each predefined cluster from the PCA step. We used the *k*-means clustering algorithm (Jain, 2010) with k = 2–20 clusters to derive the optimal k based on differences between successive values of the Bayesian information criterion (BIC) as a goodness-of-fit measure (Figure S4). The resulting DAPC is then followed by a cross-validation step via successively repeating DAPCs with an increasing number of retained PCs while keeping

TABLE 1 Summary of likelihood-ratio tests for mixed-effects models

		Initial size			Latitude (L)			Zebularine (Z)			L × Z		
Response variable	***n***	χ^2	***p***	$p_{adjusted}$	χ^2	***p***	$p_{adjusted}$	χ^2	***p***	$p_{adjusted}$	χ^2	***p***	$p_{adjusted}$
S. canadensis													
Initial size	395	–	–	–	3.02	.082	.123	**20.85**	**<.001**	**<.001**	0.00	.995	.995
Phenology 2015	388	**58.84**	**<.001**	**<.001**	**17.36**	**<.001**	**<.001**	**5.81**	**.016**	**.021**	0.38	.540	.540
Phenology 2016	381	**11.84**	**<.001**	**.001**	**11.89**	**<.001**	**0.001**	0.95	.331	.441	0.07	.797	.797
Height 2015	384	0.42	.517	.517	**6.16**	**.013**	.052	1.56	.212	.282	**4.42**	**.035**	.071
Height 2016	381	0.71	.400	.533	**7.61**	**.006**	**.023**	3.11	.078	.156	0.39	.533	.533
Total biomass 2015	390	**67.00**	**<.001**	**<.001**	**5.80**	**.016**	**.032**	1.69	.193	.193	**4.16**	**.041**	.055
Biomass ratio 2015	384	0.18	.670	.760	**8.27**	**.004**	**.016**	1.33	.248	.496	0.09	.760	.760
Ramets 2016	395	1.59	.207	.415	2.24	.134	.415	0.09	.768	.768	0.31	.581	.768
S. gigantea													
Initial size	326	–	–	–	0.04	.835	.835	**32.10**	**<.001**	**<.001**	0.21	.650	.835
Phenology 2015	288	**26.81**	**<.001**	**<.001**	0.55	.456	.608	0.88	.884	.608	0.22	.636	.636
Phenology 2016	314	**6.93**	**.008**	**.034**	0.04	.842	.850	0.04	.850	.850	0.71	.399	.799
Height 2015	286	0.57	.450	.636	1.23	.267	.636	0.51	.477	.636	0.05	.819	.819
Height 2016	313	1.95	.163	.651	0.29	.589	.671	0.18	.671	.671	0.37	.543	.671
Total biomass 2015	322	**57.34**	**<.001**	**<.001**	2.94	.087	.146	2.56	.109	.146	0.03	.854	.854
Biomass ratio 2015	284	0.05	.831	.969	0.84	.359	.717	2.11	.146	.586	0.00	.969	.969
Ramets 2016	326	0.00	.982	.999	**4.68**	**.031**	.122	0.00	.999	.999	0.14	.711	.999

Note: Analysed response variables included initial (seedling) size (number of first true leaves × length × width of the longest true leaf; cm^2), phenology (days to flowering), plant height (cm), reproductive-to-total above-ground biomass ratio, total above-ground biomass (g), and the number of ramets in the second year for *S. canadensis* and *S. gigantea*. Fixed effects included initial size (not included if response variable), latitude of source populations, zebularine treatment and the interaction of the latter two. Significant *p*-values in likelihood-ratio tests ($p < .05$) are given in bold. All *p*-values were adjusted ($p_{adjusted}$) for multiple comparisons (Benjamini & Hochberg, 1995). Detailed model parameters are given in Table S6.

all other parameters constant. In this step, the DAPCs are repeated using 90% of the data as training data to assess the proportion of successful prediction outcome of the remaining 10% to calculate the root mean square error (RMSE), an indicator of successful re-assignment to each cluster. The number of PCs with the lowest RMSE was used for the final DAPC (Figure S4). Missing alleles in the tetraploid *S. gigantea* were coded as zeros and excluded from analysis using the *recode_polyploids* function from the package POPPR version 2.8.3. For marker Sg6, a 236-bp fragment occurred in all samples and was therefore excluded from analysis. To infer population stratification with adjacent *k*-clusters and to assess the robustness of DAPC results to variation in the number of retained PCs, DAPC was repeated with $k = 2–5$ (Figure S5) and with a varying number of retained PCs (Figure S6).

To test for isolation-by-distance, we correlated the log-transformed pairwise geographical distance (km) between populations with the corresponding pairwise genetic distance (Wright's F_{ST}, standardized as $F_{ST}/(1–F_{ST})$ according to Rousset, 1997; see also Wright, 1946 and Slatkin, 1993), using the Mantel test. For the self-incompatible (Voser-Huber, 1983; Weber & Jakobs, 2005) *S. gigantea*, allele frequencies were estimated using the method of De Silva et al. (2005) developed for autopolyploid organisms under polysomic inheritance and implemented in the *deSilvaFreq* function from the package POLYSAT version 1.7-4. This function is suitable if all individuals have even-numbered ploidies and the selfing rate is known. Additionally, it is robust against underestimating common allele frequencies and overestimating rare frequencies (De Silva et al., 2005).

2.7 | Statistical analyses

As five *S. canadensis* and 10 *S. gigantea* plants died during the experiment or were replaced by buffer plants, our final 2-year data set comprised 395 plants for *S. canadensis* and 326 plants for *S. gigantea*. As some traits could not be measured on all plants (e.g., some plants did not flower), the number of plants per analysis varied (Table 1; Table S6). For all analyses of flowering phenology, we excluded plants without mature inflorescences.

We analysed flowering phenology, quantified as the number of days to flowering after June 1 with generalized linear mixed-effects models (GLMMs) suitable for count data. GLMMs were fitted with either a Poisson distribution or a negative-binomial distribution both with a log-link function. Models were analysed using zero-truncated distributions as there are no zero days-to-flowering. Phenology data from 2015 for *S. canadensis* showed signs of under-dispersion in preliminary models using either untransformed values, scaling or rank transformation (dispersion parameter $\sigma^2 < 0.6$). Data were then transformed using ordered quantile normalization (Peterson & Cavanaugh, 2019) and analysed with a linear mixed-effects model (LMM). Initial size, total above-ground biomass measured in the first year and plant height measured in both years were analysed with LMMs. The ratio of reproductive-to-total above-ground biomass, a measure of allocation to sexual reproduction, was analysed with a GLMM using a logit-link beta distribution. For both species, initial size was transformed using Yeo–Johnson transformation (YJ; Yeo & Johnson, 2000). For *S. canadensis*, plant height in 2016 and the number of ramets were transformed using ordered quantile normalization. For *S. gigantea*, plant height in 2015 and in 2016 was transformed using Box–Cox transformation (BC; Box & Cox, 1964) and YJ transformation, respectively. The type of data transformation was assessed via cross-validation (Pearson's ρ-statistic divided by its degrees of freedom) using the *bestNormalize* function in the package BESTNORMALIZE version 1.5.0. Dispersion of GLMMs (except those with a beta distribution) was assessed using the function *sigma* from the packages SPAMM version 3.0.0 and GLMMTMB version 0.2.3 and *dispersion_glmer* from the package BLMECO v1.3.

All models were fitted separately for each of the two species and included initial size (except when used as the response variable) and latitude of the source population as covariates, and zebularine treatment as a fixed factor. The covariates initial size and latitude were scaled and centred to a mean of zero and a standard deviation of one to facilitate interpretation of model coefficients. As latitude was strongly correlated with climatic variables (Method S3, Table S7 and Figure S7), we also ran models in which we replaced latitude with climatic variables (Method S4). However, we only present results of models that included latitude in the Results section (for the effect of climatic variables see Tables S8 and S9). To test whether zebularine treatment affected latitudinal clines, we included the interaction of latitude and zebularine treatment. For *S. gigantea*, we also repeated the analysis after omitting the northernmost population to evaluate the influence of this outlying population on the expression of latitudinal clines (Tables S10 and S11). Furthermore, to test whether the geographical gap in the latitudinal transect for *S. gigantea* (48.82–51.50°N; Figure 1) is reflected in phenotypic variation, we ran an additional analysis where we included the southern group (populations 1–11) versus. the northern group (populations 12–24) as an additional fixed factor (Tables S12 and S13). Finally, to test whether phenotypic variation is associated with the DAPC cluster that is most dominant in each individual, we ran a separate analysis in which we added cluster membership as a fixed factor (Tables S14 and S15).

Furthermore, models included block, source population and maternal line nested within population as random-intercept factors to account for nonindependence of plants in the same block and for nonindependence of plants from the same maternal lines nested within populations. All random effects were kept in the models (Barr et al., 2013) despite boundary (singular) fit as they were part of the experimental design and only removed if models did not converge. Models were fitted using the functions *fitme* from the package SPAMM version 3.2.0, *glmmTMB* from the package GLMMTMB version 1.0.1, and *(g)lmer* from the package LME4 version 1.1-23.

The significance of fixed model terms was tested via likelihood-ratio tests (Lewis et al., 2011; Pinheiro & Bates, 2009; Zuur et al., 2009). While we are aware that multiple testing increases the risk of type I errors, the methods available to adjust *p*-values vary considerably (García, 2004; Verhoeven et al., 2005) and have been criticized

for being overly conservative (Nakagawa, 2004; Perneger, 1998). Therefore, we based our interpretation on the unadjusted *p*-values, but we also provide *p*-values adjusted by the Benjamini–Hochberg correction (Benjamini & Hochberg, 1995; Verhoeven et al., 2005). Marginal and conditional R^2 (not available for Conway–Maxwell–Poisson, Beta and zero-truncated distributions) were calculated using the function *r.squaredGLMM* from the package MUMIN version 1.43.17. Model assumptions were checked using model diagnostics (Bolker et al., 2009; Zuur et al., 2009, 2010). Results were visualized using the R packages GGPLOT2 version 3.3.0, SJPLOT version 2.8.3, COWPLOT version 1.0.0, GGPUBR version 0.3.0, GGPLOTIFY version 0.0.5, GGTHEMES version 4.2.0 and DOTWHISKER version 0.5.0. All analyses were performed in R version 3.6.3 (R Core Team, 2020) and all data underlying the analyses are available in the Dryad repository (Eckert et al., 2020).

3 | RESULTS

3.1 | Phenotypic traits of populations from different latitudes

In the *Solidago canadensis* common-garden experiment, we found that with increasing latitude of origin plants started to flower earlier and at a smaller height, both in 2015 and in 2016 (Table 1; Figure 2b,c,e,f; Figure S8). Moreover, with increasing latitude of origin, plants of *S. canadensis* produced less above-ground biomass and invested a larger proportion of it into sexual reproduction (Table 1; Figure 2g,h; Figure S8). In contrast, the number of ramets produced by *S. canadensis* was not significantly affected by latitude of origin (Table 1; Figure 2d; Figure S8).

In *S. gigantea*, latitude of origin had no significant effect on flowering phenology, plant height, total above-ground biomass or the relative investment of biomass into sexual reproduction (Table 1; Figure 3b,c,e–h; Figure S8). However, in contrast to *S. canadensis*, the number of ramets produced by *S. gigantea* declined with increasing latitude of origin (Table 1; Figure 3d). This effect, however, became nonsignificant after removing the northernmost population (Tables S10 and S11), and also when we replaced latitude with a population-grouping factor (south versus north of the distributional gap of the species in Germany; Tables S12 and S13). These alternative analyses, on the other hand, indicated that the southern populations flowered significantly earlier than the northern ones (Tables S10–S13).

3.2 | Effect of zebularine treatment on latitudinal clines

In *S. canadensis*, the zebularine treatment reduced initial size (Figure 2a) and delayed early-stage plant height-growth (Figures S1 and S2). Furthermore, it led to a small but significant delay in the onset of flowering (Figure 2b; Figure S8). Zebularine treatment also affected the expression of latitudinal clines for plant height and total biomass in the first year of the experiment (Table 1; Figure S8). However, contrary to our expectations, zebularine-treated plants

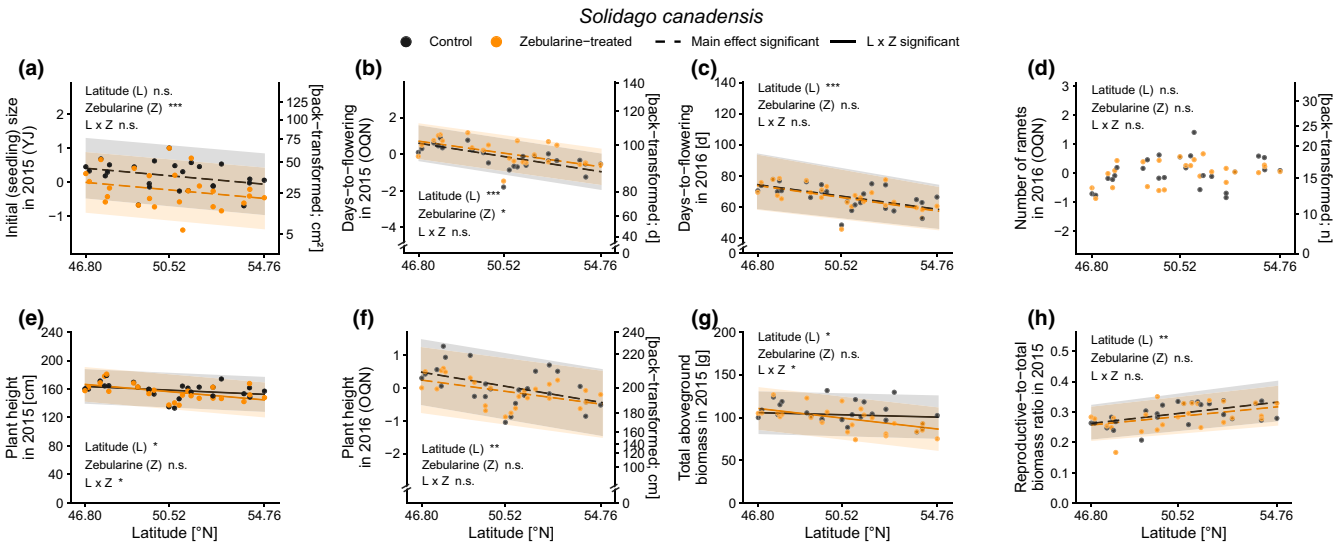

FIGURE 2 Plant traits along the latitudinal gradient for *Solidago canadensis*. Population means for control plants (black) and zebularine-treated plants (orange) related to latitude. Lines indicate the predicted marginal-effect values and their 95% confidence intervals (dashed = effect of main factors is significant; solid = effect of the interaction between latitude and zebularine treatment is significant). The effect of latitude, seed treatment and their interaction was assessed using (generalized) linear mixed-effects models and subsequent likelihood-ratio tests (Table 1; significance levels: ***$p < .001$; **$p < .01$; *$p < .05$; n.s., not significant). In cases where data have been transformed, the second *y*-axis displays the back-transformed data. (a) Initial (seedling) size in 2015 (data transformed using Yeo–Johnson transformation; YJ); (b) days to flowering in 2015 (data transformed using ordered quantile normalization; OQN); (c) days to flowering in 2016; (d) the number of ramets in spring 2016 (data transformed using OQN); (e) plant height (cm) at flowering stage in 2015; (f) plant height (cm) in 2016 (data transformed using OQN); (g) total above-ground biomass (g) harvested in 2015; (h) the ratio of reproductive-to-total above-ground biomass in 2015 [Colour figure can be viewed at wileyonlinelibrary.com]

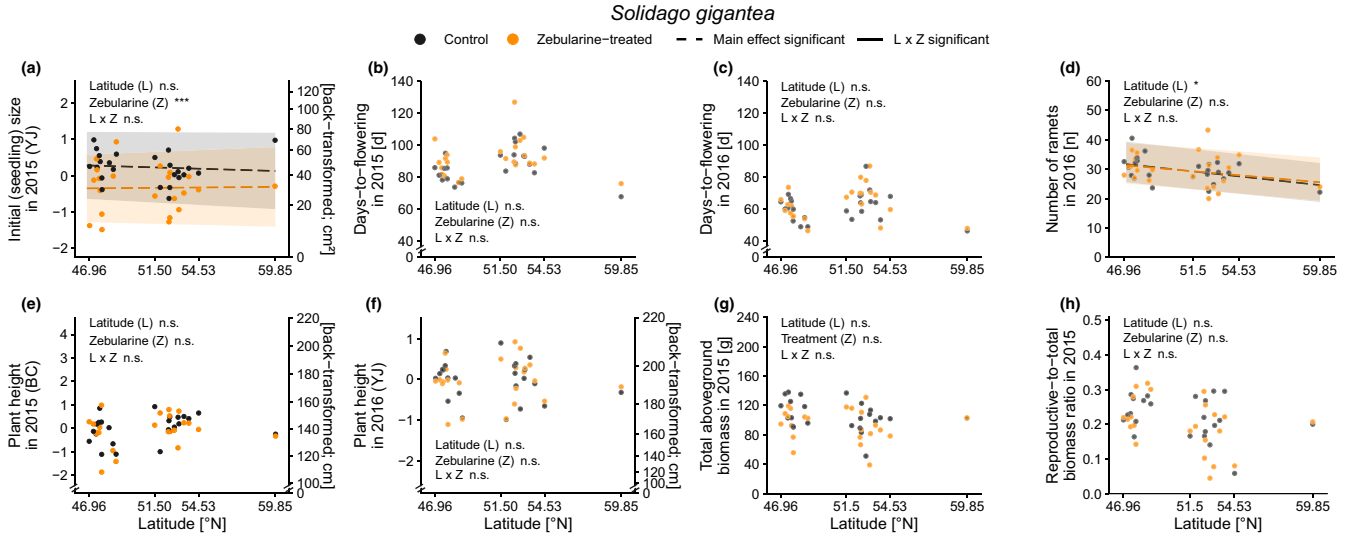

FIGURE 3 Plant traits along the latitudinal gradient for *Solidago gigantea*. Population means for control plants (black) and zebularine-treated plants (orange) related to latitude. Lines indicate the predicted marginal-effect values and their 95% confidence intervals (dashed = effect of main factors is significant). The effect of latitude, seed treatment and their interaction was assessed using (generalized) linear mixed-effects models and subsequent likelihood-ratio tests (Table 1; significance levels: ***$p < .001$; **$p < .01$; *$p < .05$; n.s, not significant). In cases where data have been transformed, the second *y*-axis displays the back-transformed data. (a) Initial (seedling) size in 2015 (data transformed using Yeo–Johnson transformation; YJ); (b) days to flowering in 2015; (c) days to flowering in 2016; (d) the number of ramets in spring 2016; (e) plant height (cm) at flowering stage in 2015 (data transformed using Box–Cox transformation; BC); (f) plant height (cm) (data transformed using YJ); (g) total above-ground biomass (g) harvested in 2015; (h) the ratio of reproductive-to-total above-ground biomass in 2015 [Colour figure can be viewed at wileyonlinelibrary.com]

showed more instead of less pronounced latitudinal clines compared to control plants (Figure 2e,g).

In *S. gigantea*, the zebularine treatment also reduced initial size (Figure 3a) and delayed early-stage height growth (Figures S1 and S2), but did not affect any of the other measured traits. Moreover, the zebularine treatment did not significantly affect latitudinal clines in *S. gigantea* (Table 1; Figure 3; Figure S8).

3.3 | Molecular genetic structure of populations from different latitudes

In *S. canadensis*, DAPC analysis gave $k = 7$ genetic clusters based on six discriminant functions and retained 15 PCs (Figure 4a). However, almost all individuals belonged to multiple genetic clusters, and the clusters did not reveal any association with latitude. In line with this, genetic distance in terms of F_{ST} did not increase with geographical distance between populations (Mantel test: $r_M = 0.013$; $p = .451$; $n = 1{,}000$; Figure 4b).

In *S. gigantea*, DAPC identified $k = 3$ clusters based on two discriminant functions and retained 25 PCs (Figure 4c). Overall, the populations could be grouped into a southern, a central and a northern genetic cluster, whereby the cluster that was dominant at intermediate latitude was also frequently found in southern and northern populations. In line with this, genetic distance in terms of F_{ST} increased with geographical distance between populations (Mantel test: $r_M = 0.288$; $p < .001$; $n = 1{,}000$; Figure 4d). DAPC cluster membership was not significantly associated with trait variation (Tables S14 and S15).

4 | DISCUSSION

In our 2-year common-garden experiments with two highly invasive goldenrod species, *Solidago canadensis* and *S. gigantea*, we tested whether plants grown from seeds showed latitudinal phenotypic clines and whether there is a potential epigenetic contribution mediated by cytosine methylation. Treatment of seeds with the demethylation agent zebularine had an overall negative effect on initial size (Figures 2a and 3a) and plant height (Table S16, Figures S1 and S2), and, for *S. canadensis*, delayed the onset of flowering in the first year (Figure 2b). By growing our plants from seeds, we showed that the latitudinal clines in phenological and performance traits previously reported for both species grown from rhizomes (Weber & Schmid, 1998) are also inherited to the sexually produced offspring generation. The clines persisted or even became slightly stronger in plants grown from seeds treated with zebularine, arguing against an epigenetic underpinning through cytosine methylation. Therefore, the observed heritable phenotypic differentiation along latitudinal gradients in the analysed traits of the two invasive goldenrod species probably has a genetic basis.

4.1 | Phenotypic latitudinal clines

In a previous common-garden experiment with plants grown from field-collected rhizomes, Weber and Schmid (1998) found that plants from northern European populations of both species flowered earlier and at a smaller size than those from southern populations. In our study, we found a similar phenotypic latitudinal cline for

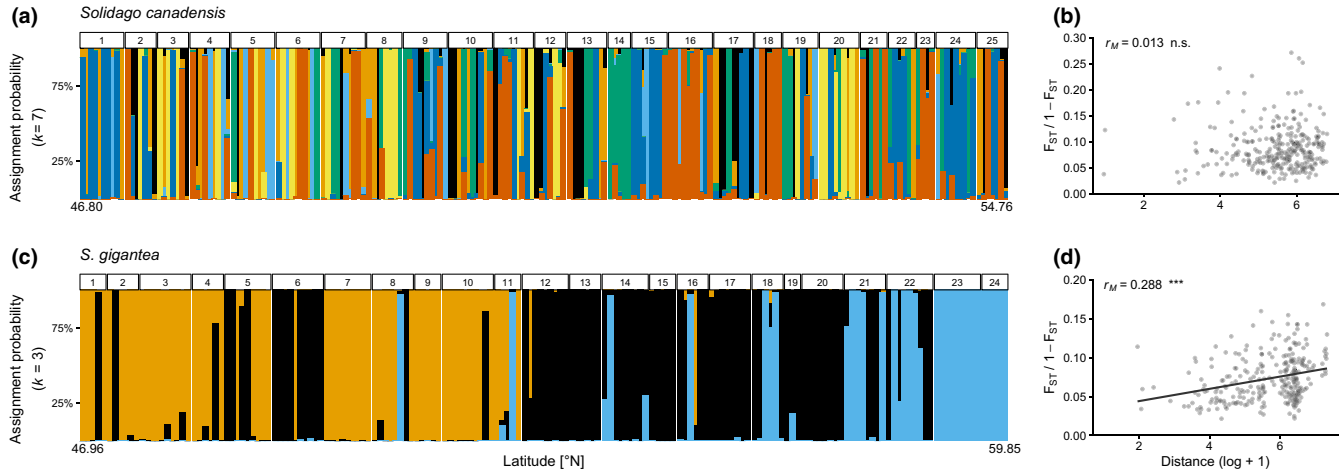

FIGURE 4 Genetic structuring in 25 *Solidago canadensis* and 24 *S. gigantea* populations. Control plants were analysed with discriminant analysis of principal components (DAPC; Jombart et al., 2010) and isolation-by-distance (Rousset, 1997) on a population-level. Isolation-by-distance was obtained by correlating the population-based pairwise fixation index F_{ST} with the logarithm of geographical distances between source populations using the Mantel test with 1,000 repetitions (r_M, Mantel's coefficient of correlation; significance levels: ***$p < .001$; n.s., not significant). Stacked bars display the probability (%) of membership (a) *S. canadensis* control plants to $k = 7$ genetic clusters based on the Bayesian information criterion (BIC) and (c) *S. gigantea* control plants to $k = 3$ genetic clusters. Scatter plots display genetic variation correlated against geographical distance for (b) *S. canadensis* ($r_M = 0.013$; n.s.) and (d) *S. gigantea* ($r_M = 0.288$; $p < .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

S. canadensis (Figure 2), but not for *S. gigantea* (Figure 3), when plants were grown from seeds instead of rhizomes. This suggests that at least for *S. canadensis* the phenotypic latitudinal clines found by Weber and Schmid (1998) are not simply parental–environmental effects carried over through rhizomes. Interestingly, while Weber and Schmid (1998) found that the size of the inflorescences decreased with latitude for both species, we found that the relative allocation of biomass to sexual reproduction actually increased for *S. canadensis* (Figure 2h). This might be because in our study the total above-ground biomass of *S. canadensis* decreased with latitude (Figure 2g), resulting in a larger relative allocation to reproduction. Thus, the results of our study are in line with those of Weber and Schmid (1998) for *S. canadensis*, but not for *S. gigantea*.

While *S. gigantea* did not show latitudinal clines with regard to flowering time, height, above-ground biomass production and relative allocation to sexual reproduction, it showed, in contrast to *S. canadensis*, a significant decrease in the production of ramets with latitude (Figure 3d). *Solidago gigantea* thus forms an exception to the pattern of increased clonality in colder environments at higher elevations and latitudes (Klimeš et al., 1997). Our unexpected finding, however, could simply reflect that the climate in our southern common garden was less suitable for plants from the north. This would also explain why plants from northern populations of *S. canadensis* grew less tall and produced less biomass than plants from southern populations in our experiment, but not when measured in their original sites (Weber & Schmid, 1998). Moreover, the previously significant decrease in the production of ramets disappeared, when the northernmost *S. gigantea* population was excluded from analysis or when genetic clusters were taken into account. However, without further experiments, we cannot exclude alternative explanations, including the possibility that the northern populations were founded from different source populations (which happened to have lower clonality) than the southern populations.

Our latitudinal transect covered a range from 46°N to 59°N and spanned about 1,800 km, similar to the previous study by Weber and Schmid (1998) on both *Solidago* species (44–61°N), and other studies on latitudinal adaptation in invasive plants (Colautti & Lau, 2015; Kollmann & Bañuelos, 2004). Both our study and that of Weber and Schmid (1998) contained a gap in the transect for *S. gigantea*, which is in line with the overall low number of records of this species in Central Germany (BfN, 2020). Latitude correlated significantly with the principal component axis (PC1) that mainly represented variation in wind speed, solar radiation and mean temperature of the driest annual quarter (Figure S7). When we replaced latitude with principal component scores of the multivariate climatic data, PC1 had strong effects on the onset of flowering, height, above-ground biomass and relative allocation to sexual reproduction in *S. canadensis* (Table S8). Moreover, for *S. gigantea*, PC1 had a significant effect on the onset of flowering and plant height in 2015, as well as the relative allocation to sexual reproduction. Surprisingly, these traits were not significantly affected by latitude alone, perhaps because for *S. gigantea* the correlation between latitude and PC1 was not as strong ($R^2 = 0.62$) as for *S. canadensis* ($R^2 = 0.84$; Figure S7). Overall, these findings suggest that climate is a strong driver of phenotypic differentiation in both *Solidago* species.

Gradual latitudinal changes in climate and in day length (Figure S9) affect flowering phenology, plant life cycles and growth (Woodward & Williams, 1987). Generally, plants at low latitudes are likely to flower earlier and grow more vigorously compared to plants at high latitudes. However, because plants at high latitudes have shorter growing seasons, there may be counter-gradient selection (Conover & Schultz, 1995) for plants that flower early at a smaller

size. This could explain why, when grown in a common garden, the *S. canadensis* plants from northern populations flowered earlier and at a smaller size than the plants from southern populations. While such climatic–ecotypic differentiation has since long been shown to be adaptive in other species, such as in *Achillea* spp. (Hiesey et al., 1942) and *Bouteloua curtipendula* (Olmsted, 1944), this remains to be tested explicitly for our *Solidago* species.

4.2 | Effects of the demethylation agent zebularine

Our study is the first common-garden study that used a demethylation agent in an attempt to experimentally disentangle epigenetic and genetic contributions to phenotypic variation in offspring of invasive plants sampled along a latitudinal gradient. Demethylation agents have been used by previous studies on the role of epigenetics in an ecological and evolutionary context (Bossdorf et al., 2010; Herden et al., 2019; Verhoeven & van Gurp, 2012).

Zebularine reduces cytosine methylation in a dose-dependent manner and may cause side-effects, although it has been found to have a longer half-life and fewer side-effects than, for example, 5-azacytidine (Baubec et al., 2009; Cheng et al., 2003). We applied a zebularine concentration of 25 μm, which was the highest concentration at which *S. gigantea* seedlings were not visibly affected in a pilot experiment testing a range of concentrations (0, 12.5, 25, 50, 100 and 200 μm; see Method S1 and Figure S10). In the common-garden experiment, we found that zebularine-treated plants of both species had smaller initial sizes (Figures 2a and 3a) and a delayed height growth compared to the control plants (Figures S1 and S2). *Arabidopsis thaliana* and *Medicago sativa* showed similar delays in plant growth at zebularine concentrations of more than 20 μm (Baubec et al., 2009). On the other hand, Wilschut et al. (2016) found no negative growth effects treating seeds of asexual lines of *Taraxacum officinale* at comparatively low concentrations (1 and 10 μm, respectively) of agar-dissolved zebularine. Although we have not performed a comparative assessment of methylation levels between treated and untreated plants, we assume that the initial reduction in seedling size and plant growth indicates that our treatment with 25 μm zebularine was probably effective. However, we strongly recommend that future studies on experimental demethylation quantify the effectiveness of the used chemical by measuring the reduction in global methylation levels.

If phenotypic differentiation in the two goldenrod species was entirely or partly caused by changes in cytosine methylation patterns, and under the assumption that the zebularine treatment was effective in reducing genome-wide methylation levels, one would expect the latitudinal clines in flowering phenology and fitness-related traits to disappear or become weaker in plants treated with the demethylation agent zebularine. We found that zebularine-treated *S. canadensis* plants flowered slightly, but significantly, later than untreated plants in the first year. However, the zebularine treatment did not remove any phenotypic latitudinal clines (Figures 2 and 3). On the contrary, it even resulted in slightly steeper clines for *S. canadensis* in plant height and total above-ground biomass (Figure 2e,g). This suggests that, although our demethylation treatment did not remove latitudinal clines in our experiment, it may still have contributed to unmasking cryptic genetic variation that otherwise would have been silenced. For example, variation in DNA methylation levels has been shown to be involved in tagging the boundary between euchromatin and heterochromatin in *Zea mays* (Li et al., 2015; Niederhuth & Schmitz, 2017) and *A. thaliana* (Roudier et al., 2009). Furthermore, Dubin et al. (2015) found that so-called gene-body methylations (GbMs) in *A. thaliana* were significantly correlated with latitude; that is, accessions from colder environments contained a higher number of GbMs, and that these may contribute to local adaptation. GbMs are cytosine-related gene methylations (i.e., they occur at CG sites) that are characterized by depletion of methylation levels at the gene's regulatory sites and are mostly associated with housekeeper genes (Bewick & Schmitz, 2017). Although it remains to be tested whether and how such methylation-based variations in gene-regulatory mechanisms might affect the analysed traits of our study species, the limited effects of our demethylation treatment on trait variation fits the idea that DNA methylation contributes much less to variation in gene expression than genetic mechanisms such as single nucleotide polymorphisms (e.g., Meng et al. (2016).

It is challenging to quantify epigenetic versus genetic contributions to phenotypic variation (Herman & Sultan, 2016), particularly because there are multiple epigenetic mechanisms, such as DNA methylation (Bewick & Schmitz, 2017; Finnegan et al., 1998; Herman & Sultan, 2016), histone modification (Bastow et al., 2004; Zhang et al., 2007) and small regulatory RNAs (Castel & Martienssen, 2013; Matzke & Mosher, 2014; Morgado et al., 2017). Furthermore, many epigenetic mechanisms have been shown to be dependent on genetic loci associated with adaptive mechanisms (Banerjee et al., 2019; Cortijo et al., 2014; Dubin et al., 2015; Nicotra et al., 2010). We focused on cytosine methylation, because this is the most thoroughly studied epigenetic mechanism to date, and has been shown to have transgenerational heritability and to play a role in adaptation to environmental stress (Hawes et al., 2018; Herrera & Bazaga, 2010; Herrera et al., 2014). Studies of epigenetic effects in nonmodel organisms have mostly focused on populations in contrasting natural environments (Gao et al., 2010; Herrera & Bazaga, 2016; Lira-Medeiros et al., 2010), and on apomictic or asexual plant species, such as *Taraxacum officinale* (Verhoeven & van Gurp, 2012; Wilschut et al., 2016) and *Festuca rubra* (Münzbergová et al., 2019). There is, to the best of our knowledge, only one other study that has addressed epigenetic latitudinal variation. Preite et al. (2015) assessed methylation states in offspring of the apomictic species *T. officinale* collected along a latitudinal gradient in Europe, and found that the investigated regions differed in epigenetic variation based on methylation-sensitive amplification length polymorphism (MS-AFLP) markers, but also due to genetic AFLP variation. Note that all studies mentioned above did not analyse entire genomes, and can therefore not exclude that some of the apparent epigenetic effects actually have a genetic cause. It thus remains challenging to separate epigenetic from genetic contributions to latitudinal clines.

4.3 | Molecular genetic differentiation

In *S. canadensis*, our clustering analysis of microsatellite variation revealed seven genetic clusters, but these did not correspond to different populations or groups thereof, and most of the genetic clusters were represented along the entire latitudinal range (Figure 4a). In other words, we found considerable genetic variation but did not find any molecular genetic structure for *S. canadensis* along the sampled latitudinal gradient. Letters document that gardeners in Europe, and particularly in England, repeatedly received *Solidago* seeds (along with soil to promote their acclimatization to the new area) from early settlers in North America over a long period of time (Wulf, 2009); this could explain the relatively high degree of genetic variation. Nevertheless, genetic variation of *S. canadensis* is still lower in invaded areas than in its native range (Alexander, Poll et al., 2009).

The frequent representation of multiple genetic clusters within single individuals indicates that there has been frequent admixture. This might have happened prior to the establishment of wild populations (see Dlugosch & Parker, 2008). However, as the seeds of *Solidago* species are light and have a pappus, and thus can be dispersed over long distances by wind (Melville & Morton, 1982), another likely scenario is that admixture followed after major gene flow among populations along the sampled gradient in Central Europe. Admixture may facilitate the spread of alien species by increasing the overall genetic variability and fitness (van Kleunen, Röckle et al., 2015; Rius & Darling, 2014). A high degree of admixture has also been shown for invasive *S. canadensis* populations in China (Zhao et al., 2015) and Japan (Sakata et al., 2015). So, while there is considerable variation in presumably neutral microsatellite loci in *S. canadensis* in Central Europe, it does not show any latitudinal population structure, probably as a consequence of extensive admixture. Combined with the fact that latitudinal clines in flowering phenology and growth were not removed by demethylation, these results might suggest that selection resulted in latitudinal genetic variation at loci of adaptive significance.

In contrast to *S. canadensis*, *S. gigantea* showed a clear genetic structure along the sampled latitudinal transect. We detected three genetic clusters, and the populations could roughly be grouped into a southern, a central and a northern genetic cluster (Figure 4c). In line with this, we also found that genetic differentiation between populations correlated positively with geographical distance (Figure 4d). Interestingly, although Schlaepfer et al. (2008) found isolation-by-distance for chloroplast-DNA variation in the native North American range of *S. gigantea*, they did not find it in the European range. This discrepancy probably reflects the scale-difference between this broad Europe-wide study and our more fine-scale latitudinal transect. *Solidago canadensis* and *Solidago gigantea* both produce large amounts of lightweight, wind-dispersed seeds and are outcrossing (Alexander, Naylor et al., 2009; Weber & Jakobs, 2005), but, in contrast to *S. canadensis*, *S. gigantea* is restricted to more moist and wet habitats, such as fen meadows (Weber & Jakobs, 2005)—ecosystems that have declined in area and frequency in Central Europe. Apparently, this limits gene flow at the scale of our sampled transect, and may also explain the gap in the distribution of *S. gigantea* in Central Germany (Figure 1; also see Weber & Schmid, 1998).

4.4 | Conclusions

Although epigenetic variation was shown to contribute to adaptation in *A. thaliana*, its broader significance remains unclear (Schmid et al., 2018). Recent literature has shown that DNA methylation appears to have only little influence on variation in gene expression (Meng et al., 2016; van Moorsel et al., 2019), and might largely reflect genetic differences (van Moorsel et al., 2019). In line with this, we found no evidence that latitudinal clines in two invasive species, *S. canadensis* and *S. gigantea*, can be explained by changes in cytosine methylation. We cannot exclude other potential epigenetic mechanisms, for example through the interplay with cryptic genetic variation (Gibson & Dworkin, 2004; Kalisz & Kramer, 2008; Zabinsky et al., 2019) or through shifts in the boundaries between euchromatin and heterochromatin (Li et al., 2015; Roudier et al., 2009), but, based on our findings, it seems most likely that genetic mechanisms underlie the latitudinal clines.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

M.v.K., J.J. and M.S. designed the study. S.E. and J.H. performed the research and collected the data. S.E. analysed the data with input from M.v.K. and M.S. All authors contributed substantially to the interpretation of the data. S.E. wrote the first draft of the manuscript and all authors contributed substantially to the revisions. All authors have approved the final manuscript draft for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Dryad repository (Dryad https://doi.org/10.5061/dryad.rr4xgxd6s; Eckert et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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