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ORIGINAL ARTICLE

Epigenomic response to insect herbivory in Lombardy poplar: assessing geographic variation in DNA methylation changes

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Abstract

DNA methylation is a key epigenetic mechanism in plants, reported to be involved in regulating responses and local adaptation to abiotic and biotic stressors that could be particularly beneficial for long-lived tree species. Given that climate change is predicted to promote insect outbreaks, epigenetic studies of herbivory responses in trees may be vital for forest management and conservation. Here, we study changes in DNA methylation in response to herbivory in the clonal tree *Populus nigra* cv. 'italica' (Lombardy poplar). Cuttings were collected from three distant geographical locations (Spain, Italy, Poland), clonally replicated and grown in a common environment. We used reduced representation bisulfite sequencing to assess methylation changes between three experimental categories: (i) insect and (ii) artificial herbivory, and (iii) undamaged plants. Our results indicate that DNA methylation in poplars from different provenance, despite being genetically very similar, responded differently to experimental herbivory with plant origin accounting for 17.2% of the variation across CG, CHG, and CHH contexts. Both insect and artificial herbivory induced a significantly higher response in CHH context. When we explore loci with contrasting methylation levels (differentially methylated cytosines, DMCs), Spanish poplars were the most responsive to insect damage while Polish trees to artificial herbivory. DMCs were primarily located in gene bodies and their flanking regions (CG, CHG) or intergenic regions (CHH). Further, about one-third of DMCs obtained in CHH context were associated with transposable elements (TEs). Our findings suggest that for a better understanding of the role of the epigenetics in plant responses to herbivory we may need to take into account history of exposure, and highlight the importance of incorporating epigenetic knowledge to manage natural forests and tree plantations to help them cope with their environmental challenges.

Keywords Biotic stress · Differentially methylated cytosines · Jasmonic acid · *Lymantria dispar* · Poplars · Reduced representation bisulfite sequencing

Introduction

Forests occupy large areas in Europe, where canopy trees provide food and shelter for numerous animals, fungi, and microorganisms; act as valuable understory habitats for a diverse community of plants; are key actors in interspecific interactions; and provide multiple ecosystem services (see e.g., Basile et al. 2020; Eberl et al. 2020). Climate change is predicted to impact on European forests but the magnitude of such effects will vary geographically, because bioclimatic zones in Europe differ both in their limitations for tree growth and in the expected shifts in temperature and drought risk (Intergovernmental Panel On Climate Change (IPCC) 2023; Lindner et al. 2010). Changes in climate are also causing shifts in the distribution range of insect species and the risk for insect outbreaks is predicted to increase (Bebber 2015; Canelles et al. 2021; Harvey et al. 2020; Lindner et al. 2010). The consequences of those outbreaks will depend on both intrinsic and extrinsic factors. For instance, it is well-established that some plants are able to change

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leaf quality in response to herbivory (e.g., Havill and Raffa 1999) and, undergo both within-generation plasticity and/or transgenerational defence induction. That in turn, improve either growth or defence against herbivory and thus being able to at least in part buffer the consequences of defoliation (Holeski 2007; Holeski et al. 2012, 2013). Understanding the mechanisms underlying the induced response to herbivory in trees, with their characteristically longer life spans, and how they may vary geographically could be particularly useful for assessing the fate of trees and forests under climate change (Aitken et al. 2008).

Epigenetic regulation has emerged as a significant element in plant adaptation to heterogeneous environments and stress response (Richards et al. 2017). In particular, DNA cytosine methylation is an epigenetic mark that is involved in determining to what extent plants can modify their features in response to biotic and abiotic stresses (Chang et al. 2020; Ramos-Cruz et al. 2021; Zhang et al. 2018). In plant genomes, DNA cytosine methylation occurs in three sequence contexts (CG, CHG, and CHH, where H = A, C, or T), each one maintained or established de novo through different enzymatic pathways, and featuring distinct genomic locations, inheritance patterns and reversibility (Hofmeister et al. 2020; Zhang et al. 2018). Symmetric methylation in CG sequence context is maintained during DNA replication because hemi-methylated CG sites are recognized by enzymes that catalyze CG methylation on the newly synthesized strand by way of 'template copying', it is often located in gene bodies (gbM), and it has been reported to be stable across generations (Dubin et al. 2015; Niederhuth et al. 2016; Yao et al. 2021). In *Arabidopsis*, gbM is typically found in moderately and constitutively expressed housekeeping genes, although its biological significance is still uncertain (Niederhuth et al. 2016; Muyle et al. 2022). DNA methylation in CHG and CHH sequence contexts is preferentially targeted by de novo methylation pathways, with CHH being less stable along cell division, and it is predominantly found in repetitive sequences and transposable elements (TEs), which are more abundant in species with larger genomes (Zemach et al. 2010; Bewick and Schmitz 2017; Kenchanmane Raju et al. 2019; Ramakrishnan et al. 2021). It is important to remark that spontaneous methylation gains and losses at individual cytosine positions in any sequence context occur at rates four to five orders of magnitude higher than genetic point mutations per unit time, whereas inheritance of such spontaneous methylation changes is mainly restricted to CG sites and to some extent to CHG (Yao et al. 2021).

Previous studies have demonstrated that plants can have functional stress memory, that is, pre-exposure to a certain stress generates a more pronounced and/or faster response against subsequent events, and that defence priming in response to herbivory is linked to epigenetic processes (Gallusci et al. 2023; Mauch-Mani et al. 2017). For example, in the annual herb *Thlaspi arvense*, plants that have been primed and elicited a second insect herbivory event exhibited changes in terms of chemical defence and in global DNA methylation levels compared to undamaged plants, and the magnitude of change varied between two ecotypes (Troyee et al. 2022). Also, in *Mimulus guttatus* parental damage produces a strong and consistent defensive response, increasing trichome production, that varies geographically according to natural levels of herbivory (Akkerman et al. 2016), it is transmitted directly to the progeny and seems to be strongly associated with the inheritance of altered methylation profiles and differentially expressed genes (Colicchio et al. 2018; Monnahan et al. 2021). Moreover, both the sequence context and the genomic location of stress-induced methylation changes seem to be relevant in developing a response, likely related to the contrasting patterns of inheritance of spontaneous methylation changes mentioned above. For instance, interactions between methylation in promoters and gbM at CG and CHG are relevant to understand gene expression in *Mimulus* (Colicchio et al. 2015) but also heritable DNA hypomethylation at selected TE-rich regions causes genome-wide priming of defence genes and high levels of disease resistance in *Arabidopsis* (Cooper and Ton 2022).

Epigenetic mechanisms should be particularly valuable for long-living organisms such as trees although current knowledge on their contribution to plant response to herbivory is still scarce (Bräutigam et al. 2013; Miryeganeh and Armitage 2025). Also, as mentioned above, most of our current knowledge on the relationships between epigenetic marks and plant response to insect attack refers to annual model plants and crops (e.g., Colicchio et al. 2018; Kellenberger et al. 2016; Rasmann et al. 2012). In this study, we analyse DNA methylation changes in response to herbivory in leaves of the Lombardy poplar (*Populus nigra* cv. 'italica' Duroi). Our study species, is a cultivated variety of the fast-growing tree *Populus nigra* L., originated from a single male mutant that has been propagated by artificial vegetative reproduction (cuttings) through Europe and worldwide since the beginning of eighteen century (Elwes and Henry 1913). These characteristics, namely clonal propagation and widespread distribution, make the species ideal to study the epigenetic mechanisms involved in response to herbivory for several reasons. First, European populations exhibit low levels of genetic variation (Díez-Rodríguez et al. 2022a), thus, it is more likely that Lombardy poplar trees would rely on epigenetic mechanisms to adapt and respond to geographic differences in environmental challenges (Peña-Ponton et al. 2024; Vanden Broeck et al. 2018). Second, previous studies in poplars have demonstrated the induction of defences after herbivory (Clavijo Mccormick et al. 2014; Havill and Raffa 1999) and distinct transcriptional responses to insect and artificial herbivory (Babst et al. 2009; Major and Constabel 2006; Philippe et al. 2010; Müller et al. 2019). Finally, its wide distribution allows us to study whether the methylation changes in response to herbivory may vary across geographic regions with contrasting environmental conditions, provided that lineage-specific and environmental-driven epigenetic marks contribute to define locally adapted ecotypes (Vanden Broeck et al. 2018; see also Sow et al. 2023).

Here, we experimentally investigate DNA methylation variation in response to herbivory in clones of *P. nigra* cv. 'italica' from three geographically distant provenances with contrasting environmental conditions (Spain, Italy and Poland). We applied two herbivory treatments, including true consumption by caterpillars of *Lymantria dispar* (Lepidoptera: Erebidae), and artificial leaf damage conducted by manual wounding combined with jasmonic acid spraying. We compared these treatments with undamaged control plants using epiGBS, a reduced representation bisulfite sequencing tool that is able to identify the sequence context and genomic location of DNA methylation at single base resolution in a reduced subset of the genome, providing a reliable and cost-efficient solution for experimental approaches (Gawehns et al. 2022; Troyee et al. 2023). Our main objective was to elucidate if and how DNA methylation responds to herbivory, with a focus on discerning common and distinct epigenetic alterations induced by the two herbivory treatments, and if the response depends on the geographical (environmental) origin in this crop variety with reduced genetic differentiation (Díez-Rodríguez et al. 2022a). We addressed the following specific questions: (1) Does genome-wide DNA methylation of Lombardy poplars originated from contrasting geographic provenances respond differently to herbivory? (2) Do insect and artificial herbivory induce comparable methylation changes? (3) Where within the genome are herbivory-induced DNA methylation variations most prevalent?

Materials and methods

Experimental design, sample collection and DNA extraction

Cuttings of 2–3 field growing adult trees from each of three distant European populations of *P. nigra* cv. 'italica' were collected in spring 2018 and transplanted into a common garden in the Marburg Botanical Garden (Germany; 50º48′02.7″N, 8º48′24.8″E) for 10 months (Díez-Rodríguez et al. 2022b). These three natural populations were located in the north of Spain (41°31′45.7″N 4°42′22.1″W), the north of Italy (44°35′22.0″N 11°03′25.8″E), and the center of Poland (52°40′17 "N 19°04′16 "E) (see Supplementary Information Fig. S1a), where they experience contrasting climates and presumably heterogeneous biotic interactions, although there is a lack of specific herbivory records. Previous studies found these three populations have very low genetic differentiation (Díez-Rodríguez et al. 2022a). From each of the garden grown trees ("ortet") we selected and cut six to nine similar sized branches ("ramets" herafter), that were ~ 30 cm long and ~ 15 weeks old ($N = 63$ ramets in total). Ramets were stored in the dark at 4°C for two weeks, afterwards they were soaked overnight in a rooting solution (50 mg/L Rhizopon AA 50 mg tablets) and planted in 2L pots, three per pot, containing a 1:1 sand:peat mixture (30% coarse sand, 20% fine sand, and 50% nutrient-poor potting soil) and 5 ml of rooting solution. Two weeks later, rooted cuttings were transplanted into individual 2L pots with the same 1:1 sand:peat mixture and regularly watered every three days. The slow-release fertilizer Osmocote Exact Mini (16 + 8 + 11 + 2MgO + TE) was added to each pot, three grams per pot, two weeks before the start of the experiment. The greenhouse conditions during the experiment were as follows: temperature (day/night) 22/18°C (± 2°C), relative humidity: 60% (± 5%), light (day/night): 16/8 h. Each of the experimental levels of treatment included three ramets per ortet. To avoid volatile organic compound (VOC) exchange, we did not group plants from various treatments together (e.g. Clavijo Mccormick et al. 2014). The ramets assigned to each treatment level were split in two trays (flood tables), randomized within the tray (once per week) and watered regularly every three days.

We employed two herbivory treatments, including true consumption by *Lymantria dispar* (Lepidoptera: Erebidae) caterpillars and artificial leaf damage conducted by simulated wounding combined with jasmonic acid (JA)-spraying, and compared them with undamaged control plants (for a graphical abstract see Supplementary Information Fig. S1b). *Lymantria dispar* is a highly polyphagous herbivore that can cause severe damage in European mixed forests (Boukouvala et al. 2022). Mechanical wounding with addition of JA and *L. dispar* consumption were expected to elicit significant responses, and by using simulated herbivory we gained precision in the application of damage and control over the introduction of material from foreign and unidentified organisms (e.g., pathogens) by live insects (e.g. Havill and Raffa 1999; Waterman et al. 2019). To control for potential positional effects, damage was always inflicted on leaves of the lower half of the main branch of each ramet, and methylation analyses were done in tissue taken from the most adjacent undamaged leaves grown in the branch's apical half. Both insect and artificial herbivory treatments were performed twice in order to induce a priming effect (first treatment) and elicit a stronger and/or quicker response during the second treatment (Mauch-Mani et al. 2017). For insect herbivory, the plants were primed with ten *L. dispar* L2 caterpillar larvae that were placed on the fully expanded leaves of the main branch of our experimental poplar ramets, which were encaged in 75*100 cm nylon mesh bags. After five days, the larvae were removed from the plants for three days to allow them to recover. For the second insect herbivory event, seven L2 instar and five L4 instar larvae were placed on lower main branch leaves, encaged in the same nylon mesh bag, and allowed to feed freely for seven days prior to the collection of leaf samples. Artificial herbivory was also conducted in the main branch and in similar locations than insects were encaged. In the priming phase, three to four leaves of the lower half of the main branch were punched with 6 to 8 holes (approximately 3 to 5 mm in diameter) per leaf. Immediately following the artificial wounding, two pumps (150 µL per pump) of a JA solution were sprayed onto the damaged leaf and three pumps were sprayed throughout the plant. This procedure was repeated three days later for the second artificial herbivory treatment by punching a total of 10–12 punched leaves per ramet and also JA spraying. In the control group, similarly positioned, well-developed leaves from each ramet's main branch were sprayed with an equivalent aqueous solution containing no JA and covered with nylon mesh bags in the same manner as the herbivory treated leaves. The experiment was concluded 17 weeks after clonal propagation.

Tissue of undamaged and completely expanded leaves of the adjacent apical half of each ramet was collected 24 h after the second herbivory event in treated plants, or after the aqueous spraying in controls, seeking to characterize systemic changes (sensu Babst et al. 2009). Throughout the duration of the experiment, these leaves were left unbagged. Five-6 discs of leaf tissue (approximately 3–5 mm in diameter) were removed using a cork borer and promptly frozen in liquid nitrogen. The vials were stored at −80 ºC until DNA extraction. The order of sampling and DNA extraction was determined by randomly selecting one sample per treatment (regardless of ortet) at a time. Frozen leaf tissue was grinded and homogenized using a Qiagen TissueLyser II equipped with two stainless steel balls (45 s at a frequency of 30.00 1/s). The NucleoSpin Plant II reagent from Macherey–Nagel was used for DNA extraction, and cell lysis Buffer PL1 (CTAB method) was used to obtain the highest possible DNA quality.

epiGBS library preparation, data processing and filtering

With a few adjustments, we followed the EpiGBS2 optimized protocol (Gawehns et al. 2022). First, the samples were randomized, and 1000 ng of DNA from each sample were digested with the restriction enzymes AseI and NsiI. The digested DNA was then ligated with hemi-methylated adapter pairs encoding barcodes of sample-specific 4–6 nucleotides. These adapters also included three random nucleotides (NNN), used during bioinformatic analysis to identify PCR duplicates as well as an unmethylated cytosine, used to annotate the Watson and Crick strands and in order to determine the bisulfite conversion rate. The samples were then multiplexed, concentrated, and smaller fragments (60 bp) removed using the NucleoSpin Gel & PCR cleaning Kit. SPRIselect™ magnetic beads (Beckman Coulter™) were used to select 300 bp DNA fragments (and lower). To get fully ligated and methylated adapters, deoxynucleoside triphosphates (dNTPs) containing 5-methylcytosine were used to repair the nicks caused by hemi-methylated adapters. We followed the protocol from the EZ DNA Methylation-Lightening kits to convert the unmethylated cytosines in the DNA to uracils, which were then substituted by thymines during the amplification stage. The transformed DNA was PCR-amplified, followed by a final clean-up and size selection. The library was then paired-end (PE 2 × 150 bp) sequenced in one lane of an Illumina HiSeq 4000 sequencer with a 12% phiX spike.

The epiGBS2 pipeline's 'reference' branch was used to process the sequencing data (Gawehns et al. 2022). All steps were implemented into a Snakemake 6.1.1 workflow (Köster and Rahmann 2012). First, PCR duplicates were removed based on the 3-random nucleotide sequence (NNN) inserted into the adapter sequences. The purpose of this step was to confirm true PCR clones so that they could be removed from the sequencing data, but not biological duplicates. Using Stacks2 software (Rochette et al. 2019) the samples were demultiplexed according to the barcodes, followed by adapter removal using cutadapt (Martin 2011). Our 63-sample library generated 740,184,096 raw sequencing reads, of which 475,316,207 (64.2%) were successfully demultiplexed and assigned to individual samples. These data are stored in ENA projects: PRJEB51623 and PRJEB51853. The bisulfite conversion rate (94.9%) was calculated by estimating the number of correctly bisulfite-converted control cytosines within the adapters (see Gawhens et al. 2022). Next, the pipeline mapped the sequencing reads of experimental data to the reference genome of *P. nigra* cv. 'italica' (available at ENA project: PRJEB44889) using the default parameters of BISMARK v0.19.0 (Krueger and Andrews 2011). Finally, strand-specific methylation and nucleotide calling were performed within the pipeline to obtain one single methylation polymorphism bed file per sample (containing rows with chromosome/scaffold name, genomic position, strand information, methylated cytosine number, unmethylated cytosine number, cytosine context (CG/CHG/

CHH), and trinucleotide context information where H are indicated by true A, T, C in the sequence).

We filtered methylation data by removing the samples with low read coverage, that is, samples that had less than 50% of the average number of cytosines present across samples. All 63 samples passed this filtering. Cytosine positions covered with less than 10 reads (10x) were removed and resulting files for each sample stored as flat file databases in R package methylKit (Akalin et al. 2012). The coverage of the Cs per sample was normalized using the *normalizeCoverage* function (method = "median") in methylKit. For each population, individual databases were then merged using the *unite* function of methylKit including all Cs present (with minimum 10x coverage) in at least 2 out of 3 of the samples per treatment group. The final methylation call dataset contained a total of 5,717,923 cytosine reads (1,630,832, 2,207,164, and 1,879,927 for Spain, Italy and Poland respectively). As the presence of C > T SNPs can impact the accuracy of detected methylation levels, we also excluded 5,691 Cs that overlapped with SNP positions that were present in the epiGBS SNP file. After removing cytosines with at least one missing value across all samples, we obtained 108,724 positions (1.9% of total) without any missing value.

Data analysis

Genome-wide methylation levels

All the statistical analyses were carried out using the R environment (R Core Team 2022). At every sequenced cytosine locus in a sample, methylation level (%) was calculated as: (methylated cytosine read count)/(methylated cytosine read count + unmethylated cytosine read count) * 100. Likewise, for each sample, we obtained genome-wide methylation levels as the average methylation levels of all cytosine loci sequenced for that sample. Given that in plants DNA methylation can occur within three cytosine contexts (CG, CHG, CHH) and that on each context has distinct properties and functions, all the statistical analyses were conducted also for each context separately.

To asses and quantify the contribution of population of origin and herbivory treatment to the variation in cytosine methylation levels, we used redundancy analysis (RDA) as implemented in the package vegan 2.6 using the function *rda* (Oksanen et al., 2022). RDA is a constrained ordination method analogous to linear regression for cases that have multiple-dependent variables (in our case, DNA methylation values for each cytosine site) and several independent variables (in our case, population of origin and herbivory treatment). These analyses were conducted using as the response matrix the DNA methylation data shared by all the samples of the three populations, i.e. without any missing values, that had 108,724 cytosine positions (11,462 in CG; 17,043 in CHG; and 80,219 in CHH). First, we assessed to what extent variation in methylation levels among samples can be explained by differences between populations and ortets using only the group of control samples ($N = 21$). With this dataset we run two different models that allowed us to test for (i) epigenetic differentiation among populations, and (ii) differentiation among ortets after adjusting for the variance explained by the populations. Next, we ran another model to test the effect of herbivory treatment on DNA methylation variation and whether this effect differed among populations for each herbivory type separately (comparing control vs. insect herbivory and control vs. artificial herbivory samples) in order to detect a potential divergence in the strength of the methylation response elicited. The significance of the models was tested using the function *anova* of the package stats.

Differentially methylated cytosines (DMCs): stress-specificity and structural annotation

Since a large number of positions were not shared among the three study populations (only ~ 25% of total cytosines in the final methylation call dataset overlapped between the three populations, see Supplementary Information Table S1), differential methylation analysis in response to experimental treatments was conducted for each population separately by using the methylation call datasets, that included Cs with a minimum coverage of 10x and present in at least 2 out of 3 of the samples per treatment group (see above). This filtering ensures the reliability and statistical power of the analysis by focusing on well-covered and consistently observed Cs across samples. On each population data, we searched for "differentially methylated cytosines" (DMCs) i.e., cytosines with a statistically significant minimum methylation difference of 10% between ramets assigned to any of the two herbivory groups in comparison to the control group. DMCs were called using a generalized linear model as implemented in the R package methylKit, including herbivory (insect or artificial) as the fixed factor and ortet as covariate. It should be noted that the testing method used by this package is able to perform only pairwise analyses (here, control vs insect herbivory and control vs artificial herbivory). The method assumes that the methylated and unmethylated counts follow a binomial distribution and the effect of the fixed factor can be estimated with a log-likelihood test for logistic regression. methylKit allows parameter adjustment to correcting by multiple testing based on q-value (q-value < 0.05), minimum percentage of methylation difference (fixed at ≥ 10%), and type of differential methylation (hypermethylation or hypomethylation, for increased or decreased methylation in experimental herbivory compared to controls). We also looked for 'stress-specific' DMCs, present in only one of the two herbivory treatments, and 'non-specific' DMCs, i.e., those shared by insect and artificial herbivory treatments.

Finally, we annotated all the herbivory induced DMCs from the former analyses to different genomic features to explore the distribution of methylation changes across the genome. We overlapped DMC location with the *P. nigra* cv. 'italica' genome (ENA project: PRJEB44889) that reports 417,754,133 bp, and provides structural annotation for 40,988 gene models, with an average length of 3175.5 bp (including exons and introns) which collectively represent 31.15% of the sequenced genome (Dubay 2024). We distinguished the following genomic features: i. the gene body, defined as the entire gene from the transcription start site to the transcription termination site, so that it includes exons and introns; ii. the promoter, defined as the region < 2 kb up-stream of the transcription start site, and iii. the downstream region, defined as the region located < 2 kb downstream of the transcription termination site. DMCs located out of those three features were classified as intergenic regions that, after accounting for the average gene length and the assigned fixed length of the two flanking regions, would be estimated as ca. 29.6% of the sequenced genome. We annotated sites following the order: gene body > 2 kb upstream of genes > 2 kb downstream of genes > intergenic regions, where a site annotated to a former feature would be excluded from subsequent annotation. In this order, if a site was annotated to "2 kb downstream of genes," it would not be annotated to the "intergenic region". Coordinates of each DMC were used to perform the BEDTools intersect command and a custom R script for annotating each genomic feature. To explore the methylation dynamics on Transposable Elements (TEs), we also searched for DMCs annotated within TEs. Transposable elements were annotated based on the TE prediction available in ZENODO (https://zenodo.org/deposit/7193978). Following the classification proposed by Peña-Ponton et al. (2024) based on TE predictions for *P. nigra* cv. 'italica' reference genome, TEs were grouped into eleven of the most important superfamilies, namely Class I LTR (copia, gypsy, unknown, SINE), or retrotransposons, and Class II DNA transposons (DTA, DTC, DTH, DTM, DTT, helitron, mite) that collectively represent ca. 34.8% of the sequenced genome (Dubay 2024). In particular, Class I LTR Gypsy elements accounted for the greatest proportion of projected TE (38%), followed by Class I LTR Copia (14%), Unknown Class I LTR (8%), Class II DNA/DTC (8%), Class II Helitron (7%) and Class II DTM (6%), other categories accounting for < 4% (Dubay 2024). A cytosine was associated with a TE when it was located inside the TE, and only the shortest predicted TE was retained. If a DMC was annotated within a TE, it will be referred to as a DMC-TE. Gene models and TE predictions used in this study were generated as part of the ongoing *P. nigra* cv. 'italica' reference genome project (PRJEB44889). With the annotation result for each population, we developed Venn diagrams to illustrate to what extent DMCs and DMC-TEs were exclusively found in one population. The short fragments interrogated with a reduced representation method such as epiGBS (less than ~ 300 bp), contain C-sites that are sparse and discontinuous in the genome, restricting the possibility to identify differentially methylated regions (DMRs) with reliable statistical confidence (Paun et al. 2019), as regularly applied in approaches that involve complete genomes, therefore no formal DMR tests were performed. It should be noted, however, that epiGBS-DMCs and DMRs obtained from Whole Genome Bisulfite Sequencing in a subsample of our study samples captured equivalent patterns of herbivory-induced methylation changes in all contexts (Troyee et al. 2023). Alternatively, aiming to look at particularly meaningful methylation changes, we ranked the DMCs and DMC-TEs and extracted the top 5% with highest hypo/hypermethylation difference between experimental groups and controls, which we termed as 'strongly responding' DMCs. Finally, we selected the group of 'strongly responding' DMCs that were annotated as "gene body", "promoter" or "downstream", and associated them with its overlapping and/or closest gene (maximum 2 kb upstream from transcription start site). The *Arabidopsis* homologue of each *P. nigra* cv. 'italica' gene was determined using BLAST reciprocal best hits (RBH) of the protein sequences (R package orthologr; Drost et al. 2015). Best hits were filtered by keeping alignments covering at least 60% of both *Arabidopsis* and *P. nigra* proteins, and minimum 60% similarity. *Arabidopsis* protein sequences were extracted from phytozome V13, and functional GO annotations were retrieved from the PLAZA 5.0 dicots database (https://bioinformatics.psb.ugent.be/plaza/). Due to the reduced number of 'strongly responding' DMCs, we were unable to find significant gene ontology (GO) terms after enrichment analysis performed using clusterProfiler v4 (Wu et al. 2021; results not shown).

Results

Genome-wide DNA methylation variation among populations and experimental treatments

Average DNA methylation level across the 63 study samples ranged between ~28.7–32.6% in CG, ~15.1–17.8% in CHG and ~7.4–8.2% in CHH. Overall, genome-wide DNA methylation exhibited a right-skewed distribution, with many cytosines showing very low methylation levels (close to 0%) and fewer showing high methylation levels (close to 100%), except for CG methylation whose distribution was nearly bimodal (i.e., more cytosines with high methylation levels; Supplementary Information Fig. S2).

Figure 1 shows variation in the average genome-wide DNA methylation per context recorded across samples from the three study populations assigned to each of the three levels of herbivory treatment. The RDA analysis including only the control samples ($N = 21$) revealed that: i. population of origin significantly explained 17.2% of the genome-wide methylation variation across samples (F = 1.87, df = 2, $P = 0.001$); and ii. ortet differences between populations explained up to 29.5% of methylation variation (F = 1.94, df = 4, $P = 0.001$).

Moreover, the RDA analysis used to test for the effect of population and herbivory treatments on genome-wide DNA methylation showed that population explained a significant proportion of the variation in DNA methylation in all sequence contexts and both herbivory types (Table 1). Finally, only in the CHH context, herbivory explained a significant proportion of the DNA methylation variation in both insect herbivory and artificial herbivory samples (Table 1) with the methylation level of control plants being, on average, lower than in plants that experienced insect or artificial herbivory (Fig. 1).

Differentially methylated cytosines (DMCs) induced by herbivory

The total numbers of DMCs, as well as the proportions of DMCs out of the total number of cytosines tested, found for each context, population and contrast (insect herbivory vs. control and artificial herbivory vs. control) are shown in Table 2. Overall, across all sequence contexts and populations, the total number of DMCs captured for insect and artificial herbivory was almost identical (11,339 and 11,319, respectively). When split by sequence context, Spanish samples had always a higher number of DMCs in response to insect herbivory in all contexts, whereas Italian and Polish samples showed higher numbers in response to artificial herbivory mainly in CG and CHG, but not in CHH. The number of DMCs captured in CHH context for both herbivory treatments was much lower than in the other two contexts. The proportion of hyper and hypomethylated DMCs was rather similar and close to 50% for CG and CHG in all three populations and both herbivory treatments (Table 2). In the CHH context, however, DMCs from all populations were relatively more hypermethylated (methylation level increased) in response to the two herbivory treatments, except for the insect herbivory treatment from Spain that showed a slightly greater proportion (61.8%) of hypomethylated DMCs.

The vast majority of DMCs (between 60 and 92%) captured in the three sequence contexts across all populations were stress-specific, that is, were induced either by insect or artificial herbivory (Fig. 2a). This was especially true in the CHH context, that showed the highest values of stress-specific DMCs in both contrasts (≥83%; Fig. 2a). In the CG context, populations showed slightly diverging trends, with ~ 50% non-specific DMCs induced by artificial herbivory in Spanish samples and ~ 40% in response to insect herbivory in Polish samples (Fig. 2a). Interestingly, most of the stress-specific DMCs across the three sequence contexts were exclusively found in one population, i.e., were stress- and population-specific, and only ~ 0.1% were shared by the three populations (6 and 12 respectively for insect and artificial herbivory; Fig. 2b).

Structural annotation of DMCs induced by herbivory

The relative distribution of the four genomic regions distinguished in the genome was estimated as ca. 31.15% for gene body, 19.6% for each of the two fixed flanking regions (promoter and downstream) and 29.65 for the intergenic regions (Dubay 2024). We found that the relative distribution of DMCs across these annotations was rather similar across populations and contexts for the two herbivory treatments (Fig. 3). In CG relative distributions of DMCs across the four genomic regions was overall similar to expected according to their relative abundance, DMCs induced by insect and artificial herbivory were predominantly located in gene body (>27%) and intergenic regions (>30%), whereas ≤ 19% DMCs were mapped to either promoters or downstream regions. This pattern was rather consistent among the three populations studied, except for the fact that the proportion of DMCs in gene bodies seemed to be slightly overrepresented in ramets from Spain compared to the other two populations (Fig. 3). In CHG, we found a lower representation in promoter and downstream regions (10–15%) that was preferentially shifted to gene bodies in Spanish ramets, intergenic regions in Italian ramets and equally to both features in Polish ramets. As regards the CHH context, a larger proportion of DMCs (≥48%) were associated with intergenic regions for the two herbivory treatments, while DMCs mapped to gene bodies were variable among populations and overall less frequent (12–26%) than in the other two sequence contexts.

DMCs induced by herbivory associated to transposable elements (DMC-TEs)

We also explored the association with transposable elements that collectively represent ca. 34.8% of the sequenced genome and found a total of 7,408 DMC-TEs (32.7% of

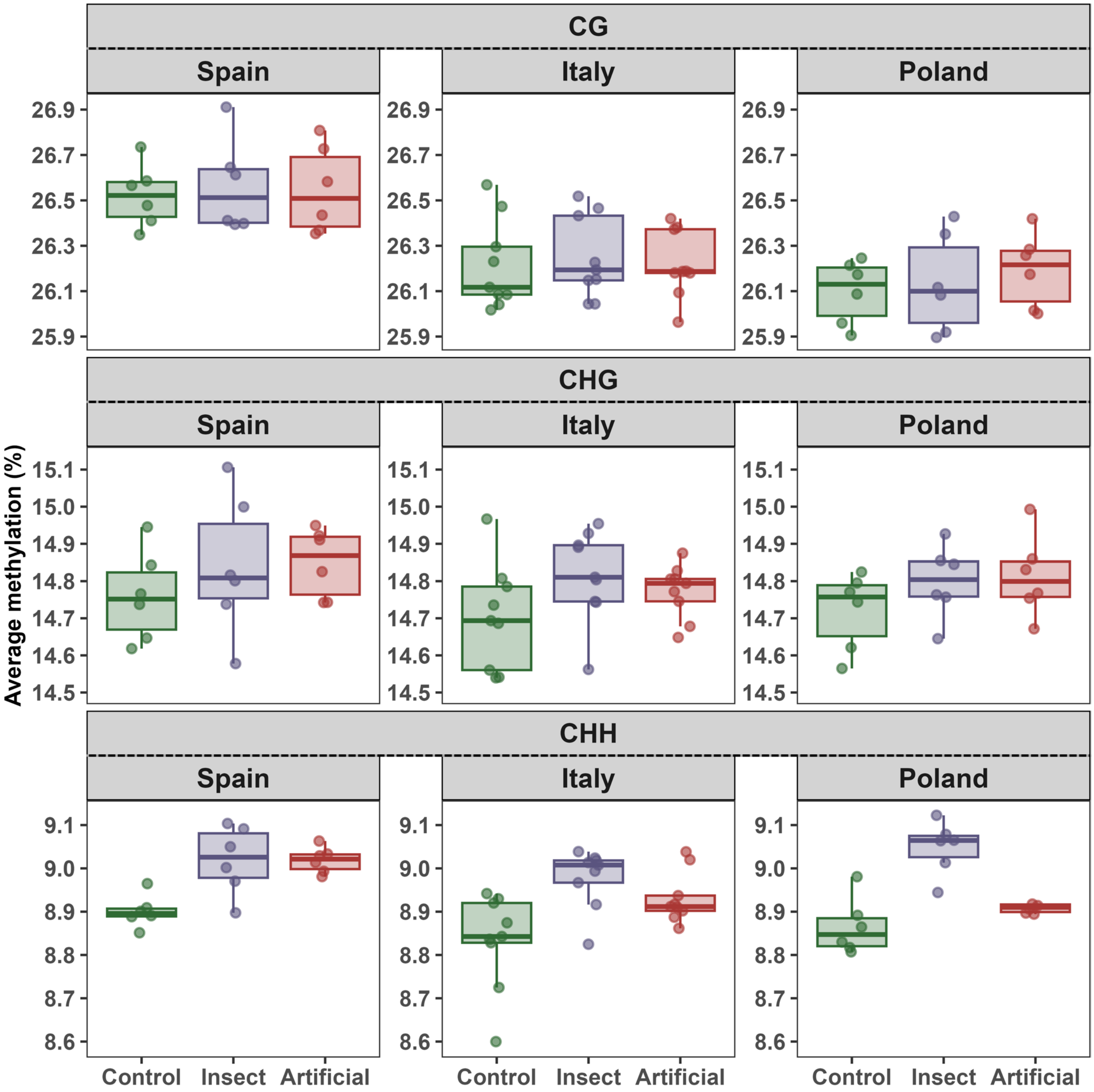


Fig. 1 Box-plots showing variation in average genome-wide DNA cytosine methylation (%) between ramets from the three study populations assigned to each of the three levels of herbivory treatment (control, insect and artificial herbivory). Each dot represents a ramet ($N = 18$, 27, and 18 for Spain, Italy and Poland, respectively). Lower and upper box boundaries are the 25th and 75th percentiles, respectively, line inside box the median, lower and upper error lines the 10th and 90th percentiles. Differences between the two herbivory levels and control undamaged plants were only statistically significant in CHH context

the total number of DMCs found across populations and treatments), including 2,470 CG-, 2,570 CHG-, 2,368 CHH-DMCs (Table 2). As a general trend, in CG and CHG contexts DMCs were less frequently annotated as DMC-TEs (~21–37%) compared to CHH context (with ~ 43–60% of DMC-TEs). On average, 58.9% of the DMC-TEs were hypermethylated and 42.1% were hypomethylated. Interestingly, DMC-TEs in the CHH context tended to be slightly more hypermethylated regardless of the type of herbivory experienced, except for insect herbivory samples from Spain (Table 2). Compared to the whole set of DMCs, a much smaller fraction of DMC-TEs were located into gene bodies (~ 14%, Supplementary Figure S3). Moreover, in all populations, we found that most DMC-TEs overlapped

Table 1 Results of redundancy analysis carried out to test for the effect of population of origin and herbivory treatment on genome-wide DNA methylation separately for the three contexts (CG, CHG, CHH) and for insect and artificial herbivory. The results of the permutation test ($N = 999$) for the significance of each of the predictors are shown. Values are in bold when $p \leq 0.05$

Herbivory	Context	Predictors	df	Variance	F	*p* (>F)
Insect	CG	Population	2	97,383	5.39	**0.001**
		Herbivory	1	5454	0.60	0.940
	CHG	Population	2	56,698	3.81	**0.001**
		Herbivory	1	6882	0.93	0.600
	CHH	Population	2	44,459	1.14	**0.001**
		Herbivory	1	22,817	1.17	**0.001**
Artificial	CG	Population	2	96,845	5.39	**0.001**
		Herbivory	1	5115	0.57	0.960
	CHG	Population	2	57,282	3.83	**0.001**
		Herbivory	1	6317	0.84	0.780
	CHH	Population	2	45,292	1.15	**0.001**
		Herbivory	1	20,338	1.03	**0.050**

Table 2 Total number of cytosines tested (Cs Tested), number of differentially methylated cytosines (DMCs; minimum coverage of 10x, 10% methylation change and q-value < 0.05) detected, number of DMCs mapped to transposable elements (DMC-TEs), and number of them that were hypermethylated (Hyper) and hypomethylated (Hypo), with their respective percentages in parentheses, in samples experiencing insect or artificial herbivory compared to control samples in the three studied populations and for each sequence context

				DMCs			**DMC-TEs**		
Context	**Population**	**Herbivory**	**Cs Tested**	**N (%)**	**Hyper**	**Hypo**	**N (%)**	**Hyper**	**Hypo**
CG	Spain	Insect	138,407	2971 (2.2)	1442 (48.5)	1529 (51.5)	627 (21.1)	305 (48.7)	322 (51.3)
		Artificial	89,888	1101 (1.2)	577 (52.4)	524 (47.6)	235 (21.3)	119 (50.6)	116 (49.4)
	Italy	Insect	171,002	1072 (0.6)	580 (54.1)	492 (45.9)	285 (26.6)	155 (54.4)	130 (45.6)
		Artificial	175,989	1681 (0.9)	739 (44.0)	942 (56.0)	453 (26.9)	206 (45.5)	247 (54.5)
	Poland	Insect	117,597	992 (0.8)	524 (52.8)	468 (47.2)	261 (26.3)	118 (45.2)	143 (54.8)
		Artificial	152,122	2459 (1.6)	1459 (59.3)	1000 (40.7)	609 (24.8)	326 (53.5)	283 (46.5)
CHG	Spain	Insect	214,576	2071 (0.9)	939 (45.3)	1132 (54.7)	591 (28.5)	298 (50.4)	293 (49.6)
		Artificial	138,552	718 (0.5)	391 (54.5)	327 (45.5)	202 (28.1)	106 (52.5)	96 (47.5)
	Italy	Insect	265,992	1035 (0.4)	632 (61.1)	403 (38.9)	363 (35.1)	245 (67.5)	118 (32.5)
		Artificial	274,209	1676 (0.6)	836 (49.9)	840 (50.1)	620 (37.0)	340 (54.8)	280 (45.2)
	Poland	Insect	182,779	724 (0.3)	408 (56.4)	316 (43.6)	250 (34.5)	134 (53.6)	116 (46.4)
		Artificial	235,699	1684 (0.7)	972 (57.7)	712 (42.3)	544 (32.3)	271 (49.8)	273 (50.2)
CHH	Spain	Insect	1,138,341	637 (0.06)	243 (38.1)	394 (61.9)	333 (52.3)	144 (43.2)	189 (56.7)
		Artificial	724,963	352 (0.05)	218 (61.9)	134 (38.1)	150 (42.6)	95 (63.3)	55 (36.7)
	Italy	Insect	1,440,489	1095 (0.08)	868 (79.3)	227 (20.7)	652 (59.5)	525 (80.5)	127 (19.5)
		Artificial	1,492,684	977 (0.07)	728 (74.5)	249 (25.5)	588 (60.2)	455 (77.4)	133 (22.6)
	Poland	Insect	956,131	742 (0.08)	610 (82.2)	132 (17.8)	321 (43.3)	248 (77.3)	73 (22.7)
		Artificial	1,253,171	671 (0.05)	444 (66.2)	227 (33.8)	324 (48.3)	202 (62.3)	122 (37.7)

(> 50%) with DNA/Helitron and LTR/Gypsy TEs (Fig. 4). The LTR/Gypsy DMC-TEs were also highly represented in all three populations, especially in the CHG and CHH contexts. Specifically, DMCs in LTR/Gypsy TEs were found in higher ratio (~ 22%) than reported in the *P. nigra* cv. 'italica' reference genome (~ 15%).

"Strongly responding" DMCs

The distribution of "*strongly responding*" DMCs (i.e., top 5% DMCs with the highest absolute methylation change across populations and treatments) along genic regions ranging from 4 kb upstream the transcription start site (TSS) to 4 kb downstream the transcription termination site (TTS) is shown in Fig. 5. The abundance of these "*strongly responding*" DMCs across populations and treatments is quite uneven, with insect and artificial herbivory treated ramets from Spain, and artificial herbivory treated ramets from Poland accounting for most of them (Fig. 5). The Italian population had the least number of DMCs with strong methylation differences between damaged ramets and controls, with almost 85% of DMCs having less than 20% methylation change. Moreover, "*strongly responding*" DMCs showed more hyper- than hypomethylation in artificial herbivory ramets from Poland, but tended to be more hypomethylated in artificial herbivory ramets from Spain (Fig. 5). When split by context, in CG the

Fig. 2 Differentially methylated cytosines (DMCs; minimum coverage of 10x, 10% methylation change and q-value < 0.05) induced by insect and artificial herbivory in the three populations studied (Spain, Italy, and Poland) and the three sequence contexts (CG, CHG, and CHH). a) Proportion of DMCs (%) that were specific to each herbivory treatment (stress-specific; light green) or common to the two herbivory treatments (non-specific; dark green); b) Venn diagram showing overlap of stress-specific DMCs between populations for insect and artificial herbivory (jointly for the three sequence contexts)

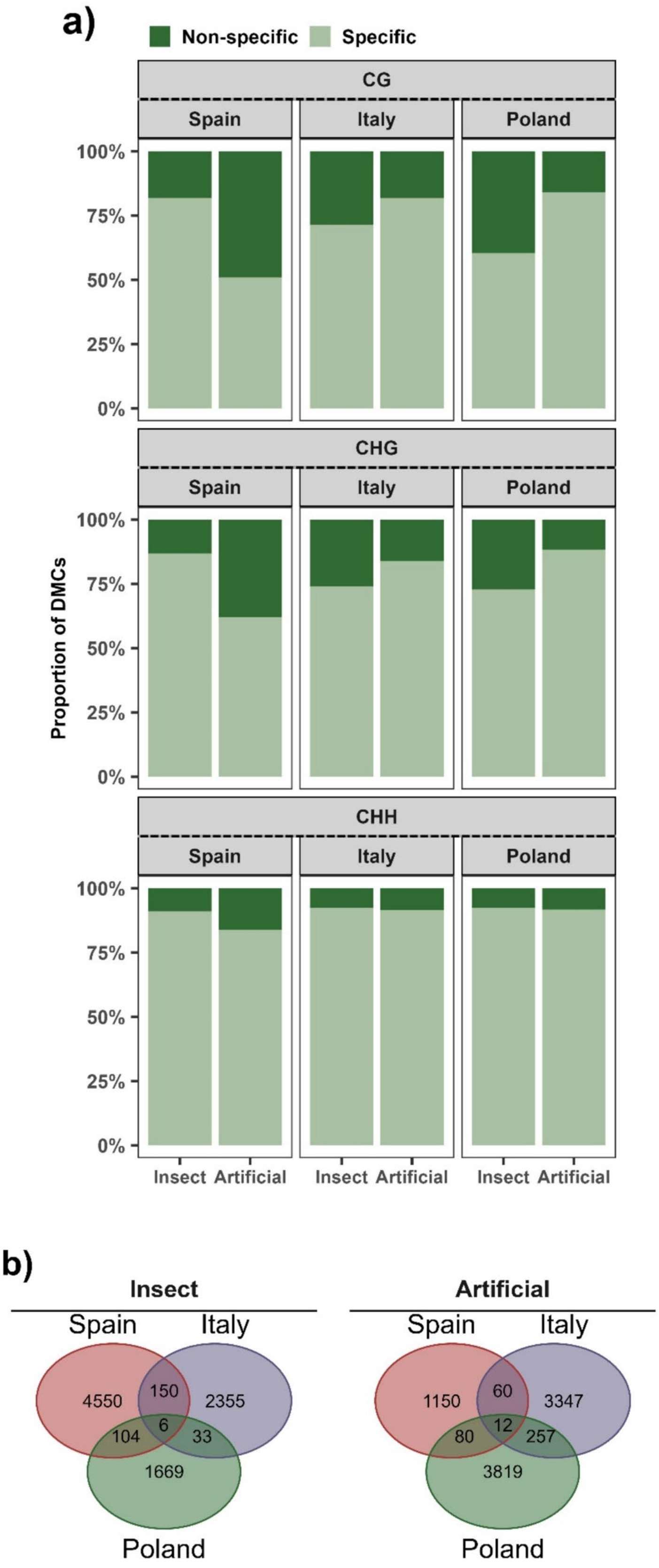


Fig. 3 Structural annotation of differentially methylated cytosines (DMCs, $N = 22{,}658$) induced by insect and artificial herbivory according to the four genomic features distinguished (promoters, gene body, downstream, and intergenic regions) in samples from Spain, Italy and Poland for the three sequence contexts

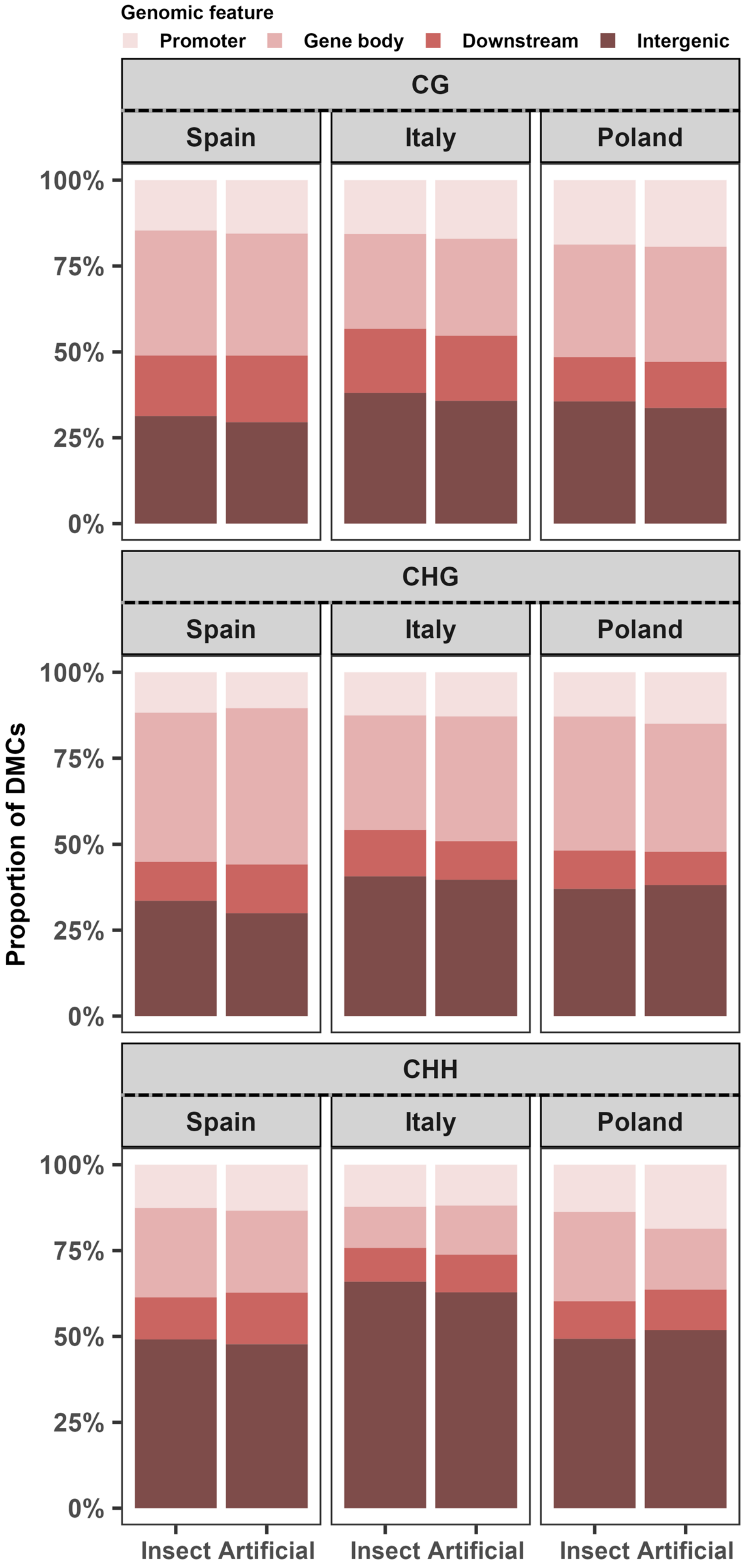
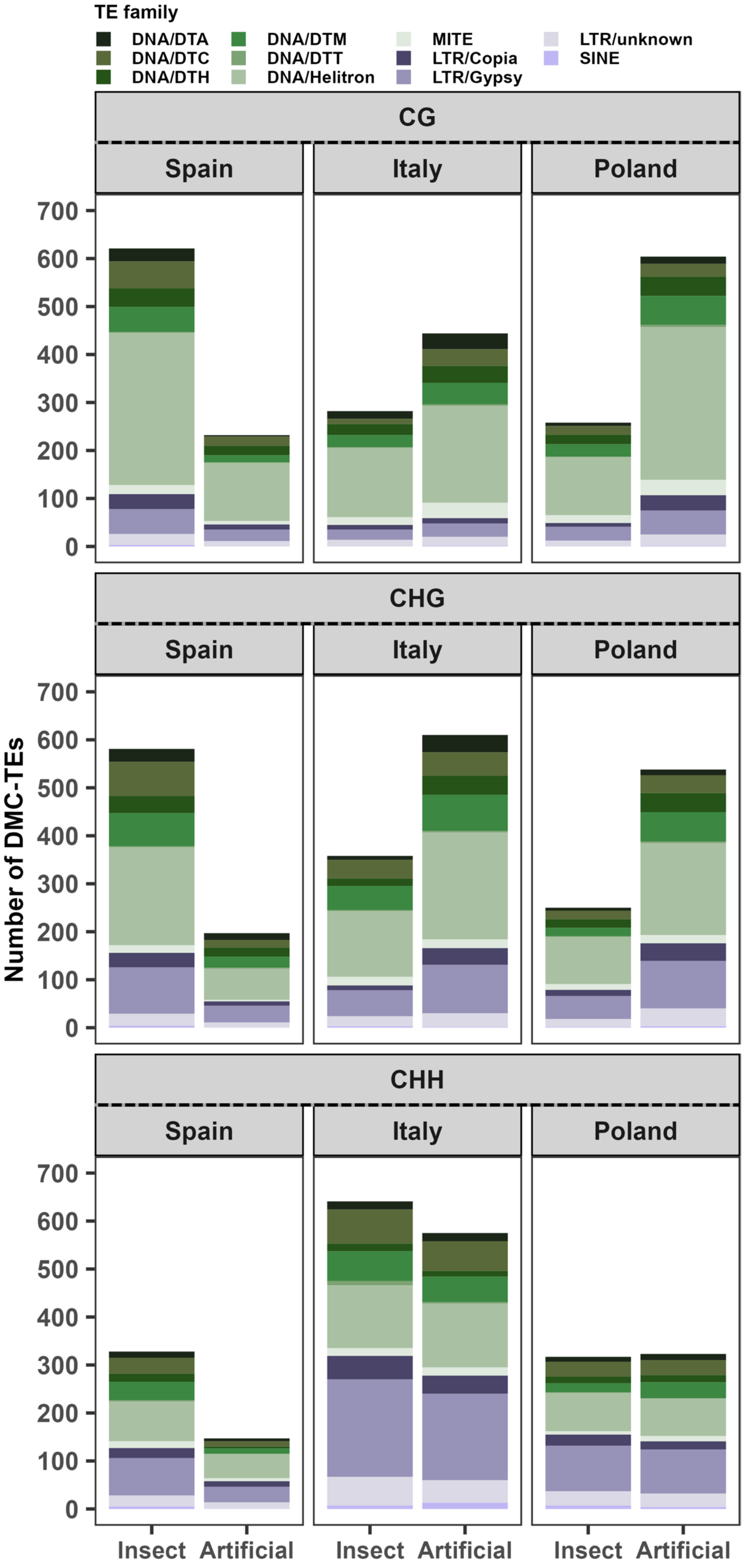


Fig. 4 DMCs mapped to transposable elements (DMC-TEs, $N = 7408$) induced by insect and artificial herbivory in samples from Spain, Italy and Poland for the three sequence contexts. Family identification of DMC-TEs have followed the classification proposed by Peña-Pontón et al. (2024) based on TE predictions for *P. nigra* cv. 'italica' reference genome. In green class II TEs, known as DNA transposons, and in purple class I elements or retrotransposons



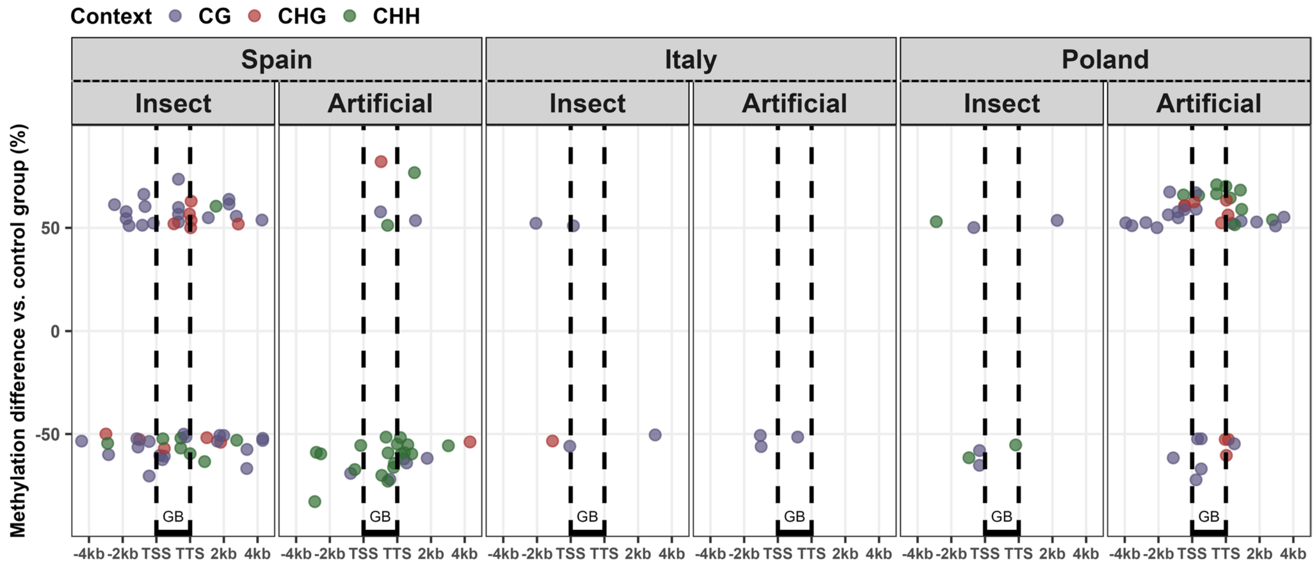


Fig. 5 Detailed genomic distribution along genic regions of the "*strongly responding*" differentially methylated cytosines (top 5% DMCs with the highest absolute methylation change across populations and treatments; $N=173$), per sequence context (CG: purple, CHG: red, CHH: green), population (Spain, Italy, Poland) and herbivory treatment (insect, artificial). Vertical dashed lines delimit gene transcription start site (TSS) and transcription termination site (TTS). The area between TSS and TTS represents the gene body (GB). Gene lengths were normalized to 2 kb

number of "*strongly responding*" DMCs was slightly higher for insect herbivory ramets from Spain and for artificial herbivory ramets from Poland. Whereas, in CHH the strongest response was found in ramets from Spain after artificial herbivory (Fig. 5). Across all populations and contexts, most of these "*strongly responding*" DMCs showed greater densities within gene bodies and the two fixed flanking regions. Interestingly, all "*strongly responding*" DMC-TEs were located in gene bodies (Supplementary Fig. S4). In total 105 of the 173 "*strongly responding*" DMCs were located in gene bodies, promoters or downstream regions, and 80 of them were associated to poplar genes that had 51 *A. thaliana* homologous genes (the complete list of GO terms obtained can be found as Online Resource 1). The annotation of these DMCs-associated candidate genes included a small number with potential roles in stress response (Online Resource 1), although these results should be taken with caution because no significant GO term was obtained after enrichment analysis (the most common Biological Process GO IDs found among the 51 *A. thaliana* homologous genes can be seen in Supplementary Information Table S2). Finally, across treatments, none of these "*strongly responding*" DMCs were shared among the three populations (Supplementary Fig. S4).

Discussion

DNA methylation is a key player in many important biological cell processes, including genomic stability, euchromatin-heterochromatin distinction, gene expression regulation, and TEs silencing (Bucher et al. 2012; Law and Jacobsen 2010; Zhang et al. 2018). It is also well-established that within plant species, individuals from distant geographic locations usually exhibit distinct DNA methylation profiles (see e.g., Bräutigam et al. 2013; Galanti et al. 2022. Particularly, in poplars, changes in DNA methylation occur during bud formation, dormancy and break associated to the seasonal leaf deciduous habit (Nunez-Martinez et al. 2024) and at a certain phenological stage, methylation profiles in leaf DNA can differ geographically (Díez-Rodríguez et al. 2022a), between nearby stands after controlling for genetic variation (see e.g. Ahn et al. 2017) and even between branches of a single individual (Yao et al. 2021). Furthermore, individual exposure to stressful conditions caused by local climate change and/or antagonistic biotic interactions, can induce epigenetic changes in a short amount of time that can be relevant for both phenotypic adaptation and genome evolution (Bucher et al. 2012; Gallusci et al. 2023; Lloyd and Lister 2022; Sow et al. 2023; Zhang et al. 2018). In particular, plant species with long generation times can undergo stress-induced changes in methylation patterns at specific genomic loci or across the entire genome, which aid in their defense mechanisms and adaptation potential (Thiebaut et al. 2019; Sow et al. 2023). Still, it is unclear how homogeneous is the epigenomic response to a certain environmental stress factor across individuals of a certain plant species (but see e.g. Herman and Sultan 2016; Alonso et al. 2025; Peña-Ponton et al. 2024). In this study, we investigate DNA methylation changes in response to experimental insect consumption and artificial leaf damage in clones of *P. nigra* cv. 'italica' propagated from trees found in three geographically distant European populations. Although we did not have previous information on defoliation records at the three tree provenances studied, we can expect some heterogeneity in past defoliation events between strands located in Spain, Italy and Poland (Charles et al. 2014). Thus, clonal propagation and a widespread distribution were ideal features to characterize genome-wide epigenetic variability in response to experimental stress in absence of genetic variation. Our study does not directly assess phenotypic susceptibility to herbivory, and therefore the observed methylation differences should not be interpreted as direct evidence of differential resistance. Instead, we highlight that even genetically identical clones may show variable epigenetic responses to the same biotic stress. In the following paragraphs, we will discuss our findings as regards common and distinct epigenetic alterations induced by either insect or artificial herbivory treatments across the three tree provenances and present the genomic location of those cytosines with extreme differential methylation between damaged and control ramets.

Genome-wide DNA methylation variation in geographically distant Lombardy poplars

In trees, both micro-environment and local herbivory can determine functional phenotypic variation in absence of genetic variation which is most likely associated to either transient or stable epigenetic variants (Díez-Rodríguez et al. 2022b; Herrera and Bazaga 2013). In poplar, DNA methylation was transmitted to the next clonal generation, but a fraction of the methylome changed relatively fast when comparing the parental individuals (i.e., ortets) with the clonal offspring (i.e., ramets) grown in a common environment (Díez-Rodríguez et al. 2022b). A Europe-wide landscape analysis, including our three study populations, confirmed limited genetic diversity and absence of genetic population structure in the Lombardy poplar, whereas epigenetic divergence was larger and to some extent correlated with geographic distance (Díez-Rodríguez et al. 2022a). Our RDA analysis showed a distinct population structure of DNA methylation in all sequence contexts, indicating a substantial epigenetic variation between the three *P. nigra* cv. 'italica' populations here studied, and additional variance between ramets in methylation levels of loci in the CG, CHG, and CHH contexts. Such methylation variability might appear stochastically, by random epigenetic changes accumulated in plants at different origins (Díez-Rodríguez et al. 2022b) and may contribute to increase phenotypic plasticity in asexuals under contrasting or stressful environments (Jueterbock et al. 2020; Verhoeven et al. 2010; Verhoeven and Preite 2014). In addition, exposures to similar stress or environmental conditions in the population of origin can result in epigenetic population structure that could influence how plant responds to a certain stress such as herbivory in subsequent seasons and generations (e.g. Conrath et al. 2015; Dowen et al. 2012; Hilker and Schmülling 2019). Altogether, in our study, we had reduced genetic variability and significant variation in DNA methylation across Lombardy poplar clones obtained from Spain, Italy, and Poland.

Insect and artificial herbivory induced methylation changes similar in sign but recorded at different genomic locations

As regards the genome-wide effect of our experimental herbivory, we found that only methylation in CHH was responsive to herbivory treatment (see also Peña-Ponton et al. 2024; Xiao et al. 2021), and changes were more significant in response to insect herbivory than artificial mimicking. DNA methylation in CHH context is established de novo and it is typically transient (Boyko and Kovalchuk 2010; Wibowo et al. 2016). Previous experimental studies also suggested that methylation in CHH is more plastic in response to several stress factors and in a variety of plant species including *P. nigra* (see e. g., López et al. 2022; Peña-Ponton et al. 2024 and references therein). In our study, DNA in leaves of damaged ramets on average exhibited higher methylation levels than DNA of control ramets, although both increased and decreased methylation were frequently found at different loci (see below).

The epiGBS methodology allowed us to explore methylation changes at single nucleotide resolution and, thus, determine not only the sign of recorded changes but also the genomic location and potential association with genes or transposable elements that might help to link mechanistic understanding in the response to the experimental factors with the ecological and evolutionary consequences (Richards et al. 2017). This technique was particularly useful in a large experimental design as the one here presented because it produces similar output than whole-genome analysis, but then at only a small part of the genome and with an affordable cost (Gawehns et al. 2022; Troyee et al. 2023). Altogether, ca. 2% of all cytosines tested were captured as DMCs, which roughly correspond to 300–3000 DMCs depending on the sequence context and paired comparison, that are within the usual range of loci detected for experimental studies conducted with reduced representation analyses in other study systems (see e.g., van Antro et al. 2023; van Gurp et al. 2016).

In any of the study populations, less than 30% DMCs were shared among the two herbivory treatments suggesting that the effects of insect consumption were not perfectly mimicked by mechanical damage and JA-spraying, as previously observed at transcription level in black poplar (Babst et al. 2009). Furthermore, in the two symmetric contexts (CG, CHG) the Spanish samples had always a higher number of DMCs in response to insect herbivory, whereas Italian and Polish samples showed higher numbers of DMCs in response to artificial herbivory. This was, to some extent, unexpected, because the artificial herbivory treatment aimed to uncover the association between priming of defenses, frequently elicited by JA (see e.g. Züst and Agrawal 2017), and changes in DNA methylation across study systems (Troyee et al. 2022). In any case, although these results need to be confirmed with more powerful tools that involve -for instance- high resolution methylome mapping, the response obtained in Lombardy poplars was not homogenous across the three tree provenances, either in magnitude or in the specific loci whose methylation was altered (see below).

Genomic locations linked to herbivory-induced methylation

Compared to other organisms, plants show broader variance in global DNA methylation levels, with non-CG methylation being frequent and predominantly found out of genes (Kenchanmane Raju et al. 2019). Thus, not surprisingly, functional consequences of methylation changes have been associated to genomic location, sequence context and the sign of change (Lloyd and Lister 2022). According to previous studies, mainly in *Arabidopsis thaliana*, TEs are highly methylated in all sequence contexts when silent and, thus, a reduction in methylation may activate TEs. However, reduced methylation in genes may increase transcription or the opposite effect depending on the sequence context and whether it is nearby the starting transcription site (CG) or at gene body for CG and CHG context (Lloyd and Lister 2022). In our study, DMCs induced by insect and artificial herbivory in CG and CHG were predominantly located in gene bodies and their 2 kb flanking regions, and increased and decreased methylation was similarly frequent. Further, DMCs in CHH were predominantly found at intergenic regions, frequently associated to TEs and overall tended to exhibit increased methylation in damaged plants, again supporting that methylation at CHH was the most responsive in our study system (see also Peña-Ponton et al. 2024 for the effect of drought). Congruently, differential non-CG methylation was found to be more strongly associated with TEs in response to leaf damage in *Mimulus* (Colicchio et al. 2018).

Epigenetic modifications such as DNA methylation affect gene expression and TE activity, but while the consequences of these methylation changes in our study on TEs are expected to be relevant, the causal link remains to be determined. In *Populus* species, TEs make up 35–40% of the genome with class I or RNA (retroelements) making up 10% and class II or DNA transposon making ca. 2–3% (Ramakrishnan et al. 2021). Our study showed that a large proportion of DMCs overlapping with TEs were associated to the LTR-Gypsy and Helitron families, mostly in the CHH context. This is partly due to the abundance of these TE superfamilies in the *P. nigra* genome, although provisional hypergeometric tests with our data pointed to significant enrichments for the LTR-Gypsy, LTR-Copia, and Helitron superfamilies (results not shown). The LTR-Gypsy are retrotransposons (class I) that mobilize through an RNA intermediate via a "copy-and-paste" mechanism (Bucher et al. 2012), and they appear to be activated by stressful conditions such as high salinity and heat in other plant species (e.g. Liu et al. 2021; Miryeganeh et al. 2022). The Helitron are DNA transposons that mobilize by a "cut-and-paste" method (class II) and are very changeable under stress, directly affecting neighboring gene transcription (stress-responsive genes) when inserted (Zhao et al. 2022).

If we pay attention to "*strongly responding*" cytosines, no overlapping existed in DMCs obtained in samples from different provenances, and very few were obtained in plants from Italy. In CG, we found a similar frequency of increased and reduced methylation changes, that are mainly associated to gene bodies either in response to insect (Spain) or artificial herbivory (Poland). In CHH, decreased methylation was more frequently observed in Spanish ramets after artificial herbivory both in gene bodies and in their flanking regions. While the functional relevance of gbM in response to stress remains unclear, it has been associated with gene expression flexibility in some studies (e.g. Bewick and Schmitz 2017) and, other studies suggest that gbM may be a passive by product of other epigenetic processes within genes (Wendte et al. 2019). Limited overlapping with functionally annotated genes precluded us to find informative associations with methylation changes elicited by herbivory. Thus, an integrative analysis of transcriptome and small RNAs might be required to better interpret our results and improve current understanding of the epigenetic mechanisms behind heterogeneous systemic induced defenses both within and across different plant species (see e.g. Babst et al. 2009; Colicchio et al. 2018, 2020; Dugé de Bernonville et al. 2020).

Conclusion

To sum up, our experimental study in Lombardy poplar showed that (i) in the control samples, a significant amount (17.2%) of variation in DNA methylation at all three contexts (CG, CHG, CHH) was explained by the population of origin; (ii) a significant increase on DNA methylation of undamaged and completely expanded leaves produced after the first priming event appeared only in CHH contexts, when the effects of insect and artificial herbivory were analyzed independently across populations. Furthermore, the combination of those two elements suggests that the response to the specific herbivory experienced may vary with plant origin because (iii) in response to insect herbivory, trees from Spain responded more than those from the other two populations, whereas for the artificial herbivory, trees from Poland were the most responsive, and Italian trees exhibited the least response in the two cases. Finally, we explored the genomic location of main methylation changes and found that (iv) DMCs were predominantly recorded in gene bodies and their flanking regions (CG, CHG) or associated to intergenic regions (CHH). Around one third of DMCs were mapped in TEs, with > 50% of these overlapping with DNA/Helitron and LTR/Gypsy TEs for both herbivory types. Overall, these results suggest that systemic response to herbivory involves changes in DNA methylation that are widespread along the genome, relatively specific to the type of herbivory experienced and geographically heterogeneous.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11295-025-01727-4.

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Data availability The bisulfite sequencing raw data is deposited in the ENA Sequence Read Archive Repository (https://www.ebi.ac.uk/ena/) under study accession numbers PRJEB51853 (https://www.ebi.ac.uk/ena/browser/view/PRJEB51853) and PRJEB51623 (https://www.ebi.ac.uk/ena/browser/view/PRJEB51623). The *Populus nigra* cv. 'italica' reference genome used is available also at ENA project PRJEB44889 (https://www.ebi.ac.uk/ena/browser/view/PRJEB44889). Extended methods (sample metadata; custom scripts, and other relevant data) will be given upon request from the corresponding author.

Declarations

Conflict of interest There are no specifc conficts of interest in this study.

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