Methylation variation promotes phenotypic diversity and evolutionary potential in a millenium-old clonal seagrass meadow

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Summary

- Evolutionary theory predicts that clonal organisms are more susceptible to extinction than sexually reproducing organisms, due to low genetic variation and slow rates of genetic evolution. However, plants that reproduce clonally are among the oldest living organisms on our planet. Here, we test the hypothesis that clonal seagrass meadows display epigenetic diversity that compensates for the lack of genetic diversity.

- In a clonal meadow of the seagrass *Zostera marina* we characterized DNA methylation among 42 shoots. We correlated methylation patterns with photosynthetic performance under exposure to, and recovery from 27°C.

- Here we show for the first time that methylation variation in clonal seagrass promotes variation in fitness-related traits of ecological relevance: photosynthetic performance and heat stress resilience. The recovered shoots memorized heat responsive methylation changes for >five weeks.

- While genotypic diversity has been shown to enhance stress resilience and invertebrate diversity in seagrass meadows composed of several genotypes, we suggest that epigenetic variation plays a similar role in clonal meadows with the potential to secure function and resilience not only of *Z. marina* plants, but of the entire associated ecosystem. Consequently, conservation management of clonal plants must include epigenetic variation as indicator of resilience and stability.

Keywords: DNA methylation, ecological epigenetics, clonality, heat stress, seagrass, *Zostera marina* (eelgrass)

Introduction

Genetic variation is considered key to long-term survival of populations (Bijlsma & Loeschcke, 2012), and is recognized as a main target for the conservation and restoration of biological diversity (Procaccini *et al.*, 2007; Evans *et al.*, 2018). In contrast, lack of genetic variation is regarded as an evolutionary dead-end (Lynch & Lande, 1998) but clonal growth challenges the direct relationship between genetic diversity and long-term survival. For example, conservation management follows the rough guideline that within a population >1,000 genetically different individuals must mate randomly to avoid inbreeding depression, and retain evolutionary potential (Frankham *et al.*, 2014).

Yet, many species produce clonal offspring that are assumed to be genetically identical. Roughly 40% of all plants can reproduce asexually (Tiffney BH, 1985), mostly by clonal growth where parental genotypes (genets) grow vegetative modules (ramets) that often remain connected via underground stolons or rhizomes.

Although clones benefit from resource sharing, niche specialization, and rapid vegetative growth (Liu *et al.*, 2016), they are predicted to survive only for short periods, and in stable environments (Silvertown, 2008). Asexual reproduction leads to the accumulation of deleterious mutations, slow rates of genetic evolution, and the lack of DNA repair mechanisms afforded by meiosis (Muller's ratchet, mutational meltdown) (Muller, 1964; Lynch *et al.*, 1993; Gabriel *et al.*, 1993). Despite asexual reproduction, many of our most important crops are clones (McKey *et al.*, 2010), including banana, garlic, hops, potatoes, and turmeric, as well as many of the earth's most invasive (Liu *et al.*, 2006; Ahmad *et al.*, 2008; Yu *et al.*, 2016), and oldest plants. For example, genets of Palmer's oak (*Quercus palmeri*) and seagrass (*Posidonia oceanica*) are estimated older than 10,000 years (May *et al.*, 2009; Arnaud-Haond *et al.*, 2012). Thus, long-term survival does not rely on sexual production.

Somatic mutations can create a certain level of genetic diversity, and may explain some evolutionary potential of clonal organisms (Whitham & Slobodchikoff, 1981; Loxdale *et al.*, 2003; Lushai *et al.*, 2003; Reusch & Boström, 2011). However, somatic mutations cannot build the genetic diversity generated by sexual reproduction, suggesting that genes are not the only components of diversity and persistence (Douhovnikoff & Dodd, 2014). Epigenetic variants may act in parallel with somatic mutations to create selectable phenotypic diversity in clonal organisms that does not depend on genetic recombination.

The definition of epigenetics is currently heavily debated (Ptashne, 2007; Greally, 2018). Here, the word 'epigenetic' implies molecular variations that do not alter the DNA sequence but have the potential to change gene expression across generations, and include non-coding RNAs (ncRNAs), histone modifications, and DNA methylation (Bossdorf *et al.*, 2008). DNA methylation is, from an evolutionary perspective, the most relevant epigenetic mechanism because it can be independent from genetic variation (Bossdorf *et al.*, 2008; Schmitz *et al.*, 2013; Kilvitis *et al.*, 2014), and transgenerationally stable (Boyko *et al.*, 2010; Verhoeven *et al.*, 2010; Ou *et al.*, 2012; Bilichak *et al.*, 2015; Williams & Gehring, 2017). DNA methylation involves the addition of a methyl-group to the C5 position of a cytosine in DNA sequence motifs (CG, CHG, and CHH in plants, where H stands for A, C, or T) (Kilvitis *et al.*, 2014). Depending on sequence context, methylation can be

associated with gene activation or silencing (Bossdorf *et al.*, 2008; Niederhuth & Schmitz, 2017). While CG methylation in gene bodies often correlates with increased gene expression, methylation in promoters and repeat regions, such as transposable elements (TEs), silences expression (Feng *et al.*, 2010; Seymour *et al.*, 2014; Dubin *et al.*, 2015; Bewick & Schmitz, 2017; Zhang *et al.*, 2018).

The methylome, or set of DNA methylation modifications in an organism's genome, can change spontaneously at a rate of 2.5×10^{-4} to 6.3×10^{-4} methylation polymorphisms per CG site per generation, which is about 7×10^{4} higher than the genetic mutation rate of base substitutions per site per generation (Schmitz *et al.*, 2011; van der Graaf *et al.*, 2015). Unlike genetic variants, methylation variants can switch state directly in response to the environment (Dowen *et al.*, 2012) and, when inherited, can create adaptive phenotypic changes within a single generation (Bossdorf *et al.*, 2008; Jablonka & Raz, 2009; Verhoeven *et al.*, 2010; Nicotra *et al.*, 2010; Hirsch *et al.*, 2012; Richards *et al.*, 2012; Douhovnikoff & Dodd, 2014; Verhoeven & Preite, 2014). That methylome variation can enhance productivity, competitive advantage, and pathogen resistance, has been shown in *Arabidopsis thaliana* plant populations (Latzel *et al.*, 2013). Moreover, methylation variation explained the rapid invasive success of the Japanese knotweed (*Fallopia japonica*) by facilitating differentiation in response to new habitats despite decreased genetic variation (Richards *et al.*, 2012). This suggests that methylation variation compensates for lack of genetic variation.

Under clonal growth, DNA methylation patterns are expected to be more stably inherited than under sexual reproduction (Verhoeven & Preite, 2014) because clonal growth circumvents epigenetic reprogramming during gameto- and embryogenesis. Methylation variation, therefore, offers potentially heritable phenotypic variability among clones (Schmid & Bazzaz, 1990; Castonguay & Angers, 2012; Massicotte & Angers, 2012), and an accelerated pathway for evolutionary change as an alternative to the slower mechanism of adaptation through selection of genetically encoded phenotypes (Feinberg & Irizarry, 2010; Herman & Sultan, 2011, 2016; Zhang *et al.*, 2013; González *et al.*, 2017). Although stable transmission across asexual generations has been shown for environment-specific phenotypes and DNA methylation patterns in clonal plants (Verhoeven *et al.*, 2010, 2018; Verhoeven & van Gurp, 2012; Vanden Broeck *et al.*, 2018), no link has been demonstrated between methylation variation and fitness-related traits. Thus, while epigenetic mechanisms have been suggested to contribute to clonal plant success (Douhovnikoff & Dodd, 2014; Verhoeven & Preite, 2014; Dodd & Douhovnikoff, 2016; Latzel *et al.*, 2016), empirical evidence is virtually lacking.

Clonal propagation is especially well-developed in aquatic plants (Barrett, 2015). Seagrasses, the only plants to inhabit the marine world, form the foundational basis of some of the most productive and highly diverse coastal marine ecosystems on the planet, and are essential for the health and abundance of economically exploited marine species (Costanza *et al.*, 1997; Larkum *et al.*, 2006; Orth *et al.*, 2006). Ecosystem services are worth more than € 16,000 ha^{-1} $year^{-1}$ (Costanza *et al.*, 1997), including nursery grounds, habitat and food for fish and invertebrates, protection of the coastline from erosion, carbon sequestration of up to 186 g C m^2 yr^{-1} (Duarte *et al.*, 2013), and nutrient fixation (Orth *et al.*, 2006; Procaccini *et al.*, 2007; Fourqurean *et al.*, 2012).

Over the last decades, losses of seagrass ecosystems have been documented worldwide due to increasing anthropogenic stressors such as invasive species, sediment and nutrient runoff, dredging, aquaculture, rising sea levels, and global warming (Orth *et al.*, 2006; Chefaoui *et al.*, 2018). Losses are expected to accelerate under projected global temperature increase, as ocean warming is considered the most severe threat among climate change factors (Repolho *et al.*, 2017; Duarte *et al.*, 2018) and seagrass meadows have tracked temperature changes in the past (Olsen *et al.*, 2004).

In this study, we characterize the functional and ecological relevance of epigenetic variation for heat stress resilience in the seagrass *Zostera marina*. *Z. marina* is the most widely distributed seagrass in the northern hemisphere, inhabiting highly contrasting habitats from sub-arctic to sub-tropical waters (Green & Short, 2004; Olsen *et al.*, 2004; Boström *et al.*, 2014). Few plants display such dramatic range in clonal diversity as *Z. marina* (Reusch *et al.*, 2000; Olsen *et al.*, 2004). Its clonal architecture varies from genetically diverse meadows with high levels of sexual reproduction, to meadows composed of a single large clone due to exclusive vegetative reproduction (Reusch *et al.*, 1999; Olsen *et al.*, 2004; Becheler *et al.*, 2010; Reusch & Boström, 2011). Clonality of *Z. marina* peaks in the Baltic Sea, where large clones were estimated > 1,000 years old (Reusch *et al.*, 1999). These meadows display remarkable phenotypic variability and persistence across space and time, under perturbations that represent environmental stress predicted for the future (Reusch *et al.*, 2018). Thus, clonal meadows (such predominated by a single genet) confound experimental results showing a positive effect of genotypic (i. e. clonal) richness on the productivity and stress resilience of *Z. marina* (Hughes & Stachowicz, 2004; Reusch *et al.*, 2005; Reusch & Hughes, 2006; Ehlers *et al.*, 2008; Hughes *et al.*, 2008).

The resilience, longevity, and adaptive potential of clonal seagrass meadows remain unknown without a fundamental analysis of their epigenetic variation and its ecological relevance. Therefore, this study tests the hypothesis that variation in DNA methylation, the most stable epigenetic

mechanism, can promote functional phenotypic diversity and, thus, may explain how clonal seagrass meadows can persist over millennia

Specific objectives were to: 1) Characterize DNA methylation variation in an ancient clonal meadow of *Z. marina* (>1,000-years-old)*;* 2) Identify the functional role of this variation in heat-stress performance; specifically how photosynthetic performance is linked to DNA methylation variation.

Materials and Methods

Sampling and cultivation

We sampled seagrass shoots from a clonal meadow (Reusch *et al.*, 1999) in the Baltic Sea (Hinderbengtsviken, Åland Islands, 60°09’50.4"N, 19°31’48.1"E) in 2015 (Fig. 1) by collecting 2-3 shoots attached to the same rhizome (ramets) every 3 meters along a 250 m transect (details in Table S1). Shoots were placed in mesh bags and transported in seawater-filled cooling boxes to the field station at Nord University, Norway. Leaf tissue of 42 shoots, randomly chosen from the 2-3 connected ramets, was flash frozen in liquid nitrogen for subsequent DNA extraction (Table S1).

Ten ramets of the same genet (Table S1) were replanted in plastic containers (10x15 cm) filled with sandy substrate from the sampling site, placed in a 1,280 L aquarium at 15°C, and illuminated with a 16:8 h light:dark cycle, and 200-220 µmol m^{-2} s^{-1} (OSRAM Fluora, 150 W). Seawater at 5.5 PSU, corresponding to the salinity at the collecting site, was obtained by mixing freshwater and natural filtered seawater at 32 PSU. The water, filled to a 40 cm level, was aerated and kept in constant motion with airstones. Once a week, 50% of the water was renewed after removing epiphytic algae.

Heat stress experiments

After two weeks of acclimation, the 10 clonal ramets were exposed to heat stress (Table S1) in climate chambers (Fitotron, weisstechnik), where they were distributed in aquaria (60 L) filled with aerated brackish water (5.5 PSU) to 30 cm. In each aquarium, 50% of the water was renewed every week. Light was kept at 240 µmol m^{-2} s^{-1} and a 16:8 h light:dark cycle. The temperature in the climate chambers was increased with a daily increment of 3°C from 15°C to 27°C, which can be lethal for *Z. marina* (Greve *et al.*, 2003), but is reached in the Baltic Sea during summer heat waves (http://www.helcom.fi/baltic-sea-trends/environment-fact-sheets/hydrography/development-of-sea-surface-temperature-in-the-baltic-sea). After three weeks at 27°C, the chamber temperature was

decreased by a daily increment of 3°C from 27°C to 15°C, after which all shoots were returned to the 1,280 L aquarium at 15°C for a recovery period of 5.5 weeks.

At three time points of the heat stress experiment (control, stress, recovery), one leaf of each shoot was excised and flash-frozen in liquid nitrogen. Control samples were collected at 15°C on the day before the temperature was increased. Stressed samples were collected at 27°C on the day before the temperature was decreased. Recovery samples were collected after the 5.5 week recovery period at 15°C.

Photosynthetic performance

At control, stress, and recovery time points, we measured for each shoot (two measurements for each of the two inner leaves) the increase in chlorophyll a fluorescence upon illumination after a 10 min dark period (OJIP curve) (Bussotti *et al.*, 2010), with a PAM-Fluorometer (FluorPen FP100, Photon Systems Instruments) using a saturating pulse of 75% at 455 nm. From the measurements, we extracted the performance index PiABS (Strasser *et al.*, 2000), reflecting the functionality of photosystem II (PSII) and photosynthetic performance in general (Živčák *et al.*, 2008; Bussotti *et al.*, 2010; Stefanov *et al.*, 2011). PiABS depends on three parameters: 1) the density of reaction centers, 2) the electron transport at the onset of illumination, and 3) the maximum energy flux reaching the PSII reaction center. We tested for correlation of PiABS values (mean values per shoot) between time points using a two-sided Pearson's product moment correlation test in R v 3.4.4 (R Core Team, 2019).

DNA extraction

All flash-frozen tissue was freeze-dried for one day, then stored at -80°C until DNA extraction. DNA was extracted with the HP Plant DNA mini kit (Omega Bio-Tek, protocol version May 2013) from ≥ 5 mg of lyophilized leaf tissue after grinding with a mixer-mill (Retsch MM 400) in 2 mL Eppendorf tubes with tungsten beads supplied (60 s at 30 Hz). We added 10 µL beta-mercaptoethanol at step 2, and equilibrated the columns at step 8 of the standard protocol. The extracted DNA was eluted in 2 x 100 µL EB buffer (Qiagen), cleaned and concentrated with the Clean and Concentrator-5 kit (Zymo Research, protocol v 1.2.1) using 15,000 g for all centrifugation steps. Three volumes of DNA Binding Buffer was added to each volume of DNA sample. After the two wash steps, the column was dried by centrifuging for 30 s. The cleaned DNA was eluted in 2 x 30 µL EB Buffer (Qiagen) at 60°C.

Clonality

In order to identify ramets belonging to the same genet, we genotyped each shoot for seven microsatellite loci (ZosmarGA2, -GA3, -GA6, -CT3, -CT12, -CT19, -CT20) (Reusch & Boström, 2011). PCR was performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Life Technologies) in two 10 µL multiplex reactions containing 2 µL cleaned genomic DNA, and 1X AccuStart II PCR ToughMix (Quanta bio). One multiplex reaction contained forward and reverse primers GA2, GA3, and GA6 at 0.5 µM each, and ran at 94°C for 4 min, followed by 30 cycles of 94°C for 60 s, 55°C for 90 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. The other multiplex reaction contained forward and reverse primers CT3 (0.5 µM), CT12 (0.3 µM), CT19 (0.3 µM), and CT20 (0.3 µM), and ran at 94°C for 3 min, followed by 35 cycles of 94°C for 60 s, 57°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 10 min.

DNA fragment lengths were determined on an ABI 3500xl Genetic Analyzer from 1 µL of 1:99 diluted PCR products mixed with 8.9 µL of HiDi Formamide (Life Technologies), and 0.1 µL of Gene Scan 500 LIZ Size Standard (Life Technologies) after 5 min denaturation at 95°C. Alleles were called with the GeneMapper v 4.1 Software (Applied Biosystems, Thermo Fisher Scientific). The shoots were assigned to multi-locus genotypes using the R package ’RClone’ (Bailleul *et al.*, 2016).

Methylome characterization

Sequencing libraries were prepared according to the MethylRAD protocol (Wang *et al.*, 2015) with few adjustments. MethylRAD is a genome-reduction method based on the methylation-dependent Mrr-like restriction enzyme FspEI that targets fully methylated CCGG and CCWGG motifs, thus capturing methylation in CG and CHG sequence contexts. MethylRAD has the potential to reveal genome-wide DNA methylation patterns that are consistent with those generated from Whole Genome Bisulfite Sequencing (Wang *et al.*, 2015). In comparison to the Methylation-sensitive AFLP method that has been popular in ecological epigenetics studies on non-model species, MethylRAD provides the advantage to allow interrogating the methylation status of annotated loci instead of anonymous markers.

First, sense and anti-sense oligos of adapters A1 and A2 (File S1) were annealed in 10 µL containing 10 µM of each oligo (Eurofins), 10 mM Tris HCl (Thermo Fisher), 50 mM NaCl (Thermo Fisher), and 1 mM EDTA (Thermo Fisher). Library preparation began with digestion of 100 ng cleaned genomic DNA at 37°C for 4 h in 15 µL containing 4 U FspEI (NEB), 1X CutSmart

Buffer (NEB), and 30X Enzyme Activator Solution (NEB). Digestion was verified on a TapeStation 2200 with a D1000 ScreenTape. Secondly, adapters were ligated to the digested fragments over night at 4°C in 26µL containing 13 µL digestion solution, 0.1 µM each of two annealed adapters, 1X T4 ligase buffer (NEB), 1.5 µM ATP (NEB) and 1040 U of T4 DNA ligase (NEB). Ligation products were amplified in 20 µL reactions containing 7 µL ligated DNA, 0.05 µM of each primer (P1 and P2, File S1), 0.3 mM dNTP, 1X Phusion HF buffer (NEB) and 0.4 U Phusion high-fidelity DNA polymerase (NEB). PCR was conducted using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Life Technologies) with 16 cycles of 98°C for 5s, 60°C for 20 s, 72°C for 10 s, and a final extension of 5 min at 72°C. The target band (approx. 100 bp) was extracted from a 2% E-Gel (Thermo Fisher). For multiplex sequencing, shoot barcodes were introduced by means of PCR. Each 20 µL PCR reaction contained 12 µL of gel-extracted PCR product, 0.2 µM of each primer (P3 and index primer, File S1), 0.3 mM dNTP, 1X Phusion HF buffer (NEB) and 0.4 U Phusion high-fidelity DNA polymerase (NEB). PCR was conducted with the same PCR cycling program outlined above. PCR products were purified using AMPURE XP beads (Beckman Coulter) using a 1.8:1 volume ratio of beads to product, and a final elution in 22 µL EB buffer (Qiagen). The purified fragments were sequenced on an Illumina NextSeq 500 (1x75bp) using a high-output flow-cell.

The sequenced reads were quality-trimmed with TrimGalore! v 0.4.1 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) by removing the adapter sequences with a stringency of 3 bp overlap, low-quality bases with a Phred score Q < 20 (99% base call accuracy), and the terminal 2 bp from both ends in order to eliminate artifacts that might have arisen at the ligation position. Quality was checked with FastQC v 0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to control for aberrant read base content, length distribution, duplication and over-representation.

The high-quality reads were mapped with SOAP v 1.11 (Li *et al.*, 2008) to 628,255 *in silico* predicted MethylRAD tags that were extracted from the *Z. marina* genome v 2.1 from ORCAE (Sterck *et al.*, 2012) with a custom python script (*InSilicoTypeIIbDigestion.py*, http://marinetics.org/2017/04/11/REdigestions.html). For mapping, we allowed for two mismatches, filtered reads with >1 N, and used a seed size of 8 bp. Based on the uniquely mapped reads, we counted the coverage of each methylated site for each shoot using htseq-count (v 0.7.2). Methylation calls were retained only for sites with ≥ 2x coverage, which reduced the false-positive rate from 1.10% to 0.23% for CG sites and from 2.50% to 0.89% for

CHG sites. False-positive rates were estimated as the percentage of methylation sites supported by at least two reads in the generally unmethylated chloroplast genome. For each shoot, raw counts were normalized to reads-per-million by dividing reads per site through the total number of reads per shoot library, times one million.

The covered (methylated) sites were annotated with the v 2.1 gff3 file from ORCAE (Sterck *et al.*, 2012), and separated into genes, intergenic regions, and TEs. TEs were located in both genes and intergenic regions, and contained repeats of the rnd-1/2/3/4 families and some selected repeats (rnd-5-family-7328, rnd-5-family-287, rnd-5-family-4856). The methylated sites were further separated into such containing CG and CHG recognition sites.

Methylation variation between the transect shoots

In order to describe the level of intra- and inter-clonal methylation differences among the 42 transect shoots, we converted the normalized counts to bivariate states (methylated/non-methylated). We then calculated the number of sites with different methylation states pairwise between all 41 ramets of the same genet, and between these ramets and the single other seven-microsatellite-genotype (transect shoot 27, Table S1).

We tested for correlations between epigenetic and physical distance among the 41 ramets belonging to the same genet with Mantel tests using the function *mantel* from the R package ’vegan’ v 1.4-2 (Oksanen *et al.*, 2019), using 1,000 permutations, and the Pearson’s product moment correlation method. Epigenetic distance was calculated as the Euclidean distance between shoots based on their coordinates in the 2-dimensional PCA (Principal Component Analysis) plot using the *dist* function of the R package ’stats’ v 3.6.0 (R Core Team, 2019). PCA was done on reads-per-million for all shoots with the PCA function of the R package ’FactoMineR’ (Lê *et al.*, 2008).

Correlation between epigenetic variation and photosynthetic performance

For the heat-stressed samples, we tested for correlations between epigenetic distances and performance differences (PiABS values) among shoots with Mantel tests using the function *mantel* from the R package ’vegan’ v 1.4-2 (Oksanen *et al.*, 2019). Performance differences at each time point (control, stress, recovery) were correlated with epigenetic distances under control conditions and at the corresponding time point. Only correlations with coefficients $R > 0.65$ and $p < 0.05$ were considered significant.

Methylome heat stress response

The methylome of each of the 10 heat-stressed shoots was characterized under control, stress, and recovery conditions (3 shoots died from the stress), and compared with the methylome of pre-acclimation samples (transect shoots) with PCA on reads-per-million data using the PCA function of the R package 'FactoMineR' (Lê *et al.*, 2008). Epigenetic distance between the samples was estimated for each sequence context (gene, intergene, TE, each in CG and CHG contexts, respectively) as the Euclidean distance in the 2-dimensional PCA plot using the *dist* function of the R package 'stats' v3.6.9 (R Core Team, 2019).

Differential methylation between control, stress, and recovery

To estimate the number of sites that changed in methylation state from control to stress and recovery conditions, we applied differential methylation analyses using the R package 'edgeR' 3.20.9 (Robinson *et al.*, 2009) within the 'SARTools' pipeline v1.6.6 (Varet *et al.*, 2016). Read counts were normalized using a trimmed mean of M-values (TMM) between each pair of samples (Robinson & Oshlack, 2010). *P*-values, indicating the significance of increased or decreased methylation, were adjusted according the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

For differentially methylated sites within gene bodies between control and recovery samples, we tested for enriched gene ontology terms of biological processes with the R package 'topgo' v 2.16.0 (Alexa & Rahnenführer, 2010) using Fisher's exacts tests. GO terms were obtained from the v 2.1 *Zostera* genome annotation from the ORCAE database (Sterck *et al.*, 2012). To reduce redundancy in the significantly enriched GO terms (p-values < 0.05), we calculated 'sim rel' scores (Schlicker *et al.*, 2006) (allowed similarity = 0.5), based on the *A. thaliana* GO-term database, using the REVIGO web server (Supek *et al.*, 2011).

Differential methylation between shoots of high and low performance

To identify how the methylome of samples with high performance differed from that of samples with low performance, we used differential methylation analyses using the R package 'edgeR' 3.20.9 (Robinson *et al.*, 2009) within the 'SARTools' pipeline v1.6.6 (Varet *et al.*, 2016). Read counts were normalized using a trimmed mean of M-values (TMM) between each pair of samples (Robinson & Oshlack, 2010). Differential methylation analysis was done only for conditions and sequence contexts where a positive correlation was found between performance differences and

epigenetic distance. For control conditions, we compared methylation levels in genes (CG regions) between the two samples of highest performance (79.1, and 13.2) and the two samples of lowest performance (63.1 and 59.2, Fig. S1). For recovery conditions, we compared methylation levels between the two samples of highest performance (79.1 and 17.1) and the two samples of lowest performance (57.1 and 59.2) in intergenic regions and TEs (only CHG regions).

For differentially methylated sites in genes (CG regions), we tested with Fisher's exact tests for enrichment of gene ontology terms of biological processes with the R package 'topgo' (Alexa & Rahnenführer, 2010). To reduce redundancy in the significantly enriched GO terms (p-values < 0.05), we calculated 'sim rel' scores (Schlicker *et al.*, 2006) (Allowed similarity=0.5), based on the *A. thaliana* GO-term database, using the REVIGO web server (Supek *et al.*, 2011).

Results

Methylome characterization

On average, 74 million high-quality reads were obtained per sequencing library, ranging from 0.7 to 151 million (Table S2). On average, 35% of the high-quality reads mapped to the *in silico* digested *Z. marina* genome, and 11% mapped uniquely (annotated reads-per-million in File S2). In total, 144,420 sites were methylated (covered by at least two reads) across all transect shoots. Across all transect shoots, 84,640 methylated CG sites and 59,780 methylated CHG sites were detected, which represents 59% and 41% of all methylated sites, respectively (Table S3). About 23% of all methylated sites fell in gene bodies (of which 41% in TEs), and 77% in intergenic regions (of which 42% in TEs) (Fig. S2). In gene bodies, 67% of the methylated sites fell in CG regions, in intergenic regions only 56%.

Methylome variation between the clonal transect shoots

Based on their multi-locus microsatellite genotype, 41 of 42 shoots sampled along the transect (not sample 27 in Table S1) belonged to the same genet (genotypes in Table S4). Methylome variation between these 41 clonal ramets exceeded the methylome shift induced by heat stress (Fig. 2), and was not higher than between shoots of different genets (Fig. 3, Table S3). Methylation differences were generally lower in CHG than in CG sequence contexts. Positive correlation between epigenetic and geographic distance at CHG sites in gene bodies ($p < 0.05$ Table S5) is here considered too weak (R=0.124) to be regarded important or representative.

Performance changes under heat stress

Photosynthetic performance (PiAbs) declined in all shoots under heat stress (Fig. 1, raw performances in Table S6). Seven of 10 shoots recovered from the heat stress, yet performances did not reach pre-stress levels. Performances under control and recovery conditions were positively correlated ($p<0.05$, $R=0.93$).

Correlation between epigenetic variation and performance

Epigenetic distances in CG gene body regions correlated positively with performance differences prior to stress (Fig. 4 a), and after recovery (stress resilience, Fig. 4 b, Table S9). Performance differences after recovery also correlated positively with stress-responsive epigenetic distances in intergenic CHG regions (Fig. 5 a), including intergenic TEs (Fig. 5 b).

Methylome heat stress response

Methylation patterns changed under heat stress and did not return to control (pre-stress) patterns during the recovery period (Fig. 6, annotated reads-per-million in File S3). More sites became hyper- than hypo-methylated in response to stress (Fig. 7). Methylation levels changed significantly at 437 sites from control to stress conditions (257 hyper- and 180 hypo-methylated), at 1788 sites from control to recovery conditions (1141 hyper-, and 647 hypo-methylated), and only at 39 sites from stress to recovery conditions (18 hyper-, and 21 hypo-methylated, Table S7). After recovery, CG methylation had been more heat-responsive in gene body regions, and CHG methylation in intergenic regions (Fig. 7).

After recovery, methylation increased in gene bodies with functions including DNA transcription and replication; catabolism of misfolded proteins, gamma-aminobutyric acid (GABA), and neurotransmitter; as well as amino acid synthesis (Table S8). DNA methylation had decreased in gene bodies with functions including transmembrane transport of cations, ions, and protons; transport of ammonium and phospholipids; localization of lipids and organelles; exocytosis; and secretion (Table S8).

Differential methylation between shoots of high and low performance

Recovered shoots of high and low performance presented highly similar methylation patterns. Only three intergenic CHG sites, and seven CHG sites in intergenic TEs were hyper-methylated in recovery samples of high photosynthetic performance (Table S10). In contrast, 89 CG gene body

sites were hyper-methylated (1 hypo-methylated) in control samples of high photosynthetic performance (Table S10). Enriched biological processes in the 89 hyper-methylated CG sites included ’light harvesting in photosystem I’ and ’protein folding’ (Table S11).

Discussion

Plant genets persisting >1,000 years challenge the direct relationship between genetic variation retained through recombination and long-term survival. Our study shows for the first time that ramets of the same seagrass genet display DNA methylation variation that is associated with phenotypic variation in the fitness-related traits of photosynthetic performance and heat stress resilience. Heat responsive methylation changes were memorized for at least five weeks in the recovered shoots. This suggests that methylation variation compensates potential evolutionary costs of clonal reproduction (Lynch *et al.*, 1993; Lynch & Lande, 1998), and contributes to the long-term survival of clonal seagrass meadows by increasing mitotically heritable and ecologically relevant phenotypic variation.

Methylome variation of functional relevance

The seagrass methylome is flexible and responds directly to environmental change, given its strong response from pre-acclimation transect samples to post-acclimation control samples within two weeks (Fig. 2). Methylome variation among ramets of the same genet resulted either from random epimutations or from microscale variations in the environment because its correlation with geographic distance between shoots was weak and, thus, with environmental factors that changed gradually along the sampled transect. Whether environmentally induced or not, methylome variation in CG gene regions was associated with pre- and post-stress performance (Fig. 4a,b) and, thus, is likely functionally relevant for thermal acclimation and stress resilience.

Although difficult to demonstrate (Crisp *et al.*, 2016), the correlations between methylome and performance differences appear to be causal, given that samples of high performance showed hyper-methylated CG sites in gene bodies with relevant functions: ’light harvesting in PSI’, ’protein folding’, ’protein refolding’, and ’misfolded or incompletely synthesized protein catabolic process’ (Table S11). These functions can prime against heat stress when increased methylation is associated with expression of the underlying genes. Indeed, while genes are generally repressed by methylated promoter regions (Lisch, 2009), gene expression is often associated with CG gene body methylation (Zhang *et al.*, 2006; Schmitz *et al.*, 2013; Yang *et al.*, 2014; Dubin *et al.*, 2015;

Bewick & Schmitz, 2017; Niederhuth & Schmitz, 2017) that prevents aberrant expression from intragenic promoters (Takuno & Gaut, 2012; Neri *et al.*, 2017).

Via association with gene expression, the observed methylome variation can create ecologically relevant phenotype variation. This supports earlier studies suggesting that DNA methylation variation enhances phenotypic plasticity in plant populations deprived of genotypic diversity: asexual dandelions (*Taraxacum officinale)* (Verhoeven & van Gurp, 2012), Lombardy poplar (*Populus nigra*) (Vanden Broeck *et al.*, 2018), Japanese knotweed (*Fallopia japonica*) (Richards *et al.*, 2012), *A. thaliana* (Latzel *et al.*, 2013; Zhang *et al.*, 2013), and the alligator weed (*Alternanthera philoxeroides*) (Gao *et al.*, 2010). Thus, our results provide a first indication that DNA methylation variation provides a non-genetic layer of ecologically relevant, and potentially selectable, phenotypic variation in clonal seagrass meadows.

Although our study is based on clonal ramets that presented identical seven-locus microsatellite genotypes, we cannot exclude the presence of undetected genetic variation generated by somatic mutations (Whitham & Slobodchikoff, 1981; Loxdale *et al.*, 2003; Lushai *et al.*, 2003; Reusch & Boström, 2011). Trans-acting SNPs appeared to explain the correlation between gene body methylation and latitude in *Arabidopsis* (Dubin *et al.*, 2015). In contrast, part of the correlation between DNA methylation with ecological factors in landscape epigenomic studies on non-model plant species could not be predicted from the observed underlying patterns of genetic relatedness (Schulz *et al.*, 2014; Foust *et al.*, 2016; Gugger *et al.*, 2016). To what degree the observed methylation variation in a plant clone is independent from low-level genetic variation remains a question of further study that captures methylation variants in parallel with SNPs. Independent from genetic variation is the methylome stress-response that provides potential for evolutionary change at time scales unattainable by somatic mutations.

Evolutionary potential of the epigenetic heat stress response

Stress recovery is a critical period during which the degree of resetting versus memory of epigenetic stress response is determined (Crisp *et al.*, 2016). *Z. marina* memorized much of the methylome stress response after a 5-week recovery period (Fig. 6). The methylation changes from control to recovery conditions were initiated in the stress phase, and then continued to diverge from control conditions, given the strong similarities in methylation profiles between stress and recovery phase (Fig. 7, Table S7). The memory of the epigenetic heat stress response is strong enough to potentially heat-harden the same generation of previously exposed shoots.

For the stress-responsive methylation changes to be evolutionary relevant, they must be stable across generations. Our study focused on a single mitotically grown generation. Across mitotically grown generations, epigenetic patterns can be expected to be more faithfully inherited than across sexual generations, because clonal growth circumvents epigenetic reprogramming during meiosis and embryogenesis (Hirsch *et al.*, 2012; Douhovnikoff & Dodd, 2014; Verhoeven & Preite, 2014; Crisp *et al.*, 2016; Latzel *et al.*, 2016). CG methylation, maintained by a homologue of mammalian DNA METHYLTRANSFERASE (MET1) (Law & Jacobsen, 2010), is likely more stable than CHG methylation, which is maintained by a dynamic process involving plant-specific CHROMOMETHYLASE and a feed-forward loop involving histone 3 lysine 9 methylation (Law & Jacobsen, 2010). Thus, we propose that memorized methylation changes, particularly changes in CG methylation that had been most prominent in gene bodies (Fig. 7), are most likely to contribute to transgenerational plasticity across mitotically grown seagrass generations, and to offer an accelerated pathway for evolutionary change (Bräutigam *et al.*, 2013; Zhang *et al.*, 2013; van der Graaf *et al.*, 2015; Platt *et al.*, 2015; Gugger *et al.*, 2016; Schmid *et al.*, 2018). Memory stability across asexual generations can only be addressed by tracking stress-induced methylation changes in new vegetatively grown shoots not experiencing the stress.

For the stress-responsive methylation changes to be adaptive, they must enhance stress performance or recovery from heat in the next generation. While heat-responsive (post-stress) methylation patterns in intergenic CHG regions, including intergenic TEs, correlated with heat stress resilience (Fig. 4a,b), we cannot tell whether these patterns will enhance resilience in the next generation. Adaptive transgenerational plasticity is most likely to evolve when the parental environment predicts the offspring environment (Laforsch *et al.*, 1999; Galloway & Efferson, 2002), and has been documented in various sexually reproducing plant species, including *Oryza* spp., *Arabidopsis*, *Polygonum* spp., and *Plantago lanceolata* (Herman & Sultan, 2011). For example, reproductive output in F3 *Arabidopsis* progeny increased five-fold under heat stress (30°C) if the F0 and F1 generations had previously experienced the same stress (Whittle *et al.*, 2009). That DNA methylation memories can mediate such adaptive transgenerational plasticity was shown in the plant *Polygonium persicaria*, in which offspring demethylation with zebularine removed the adaptive effect of parental drought in form of longer root systems and greater biomass (Herman and Sultan, 2016). Transgenerational methylation memories were further correlated with increased salt tolerance in F1 progeny of salt-stressed *Arabidopis* parents (Boyko *et al.*, 2010), a shift in flowering time in F2 and F3 progeny of *Arabidopsis* subjected to disturbance (Schmid *et al.*, 2018), and with N-deficiency tolerance in F3 progeny of N-depleted rice (*Oryza sativa*) (Kou *et*

al., 2011). Whether the stress-responsive methylation patterns in *Z. marina* are involved in the inheritance of acquired adaptive traits requires further experiments involving at least F1 progeny.

Biological functions affected by stress-responsive methylation changes suggest that the plants were investing after stress in the breakdown and repair of misfolded proteins, synthesis of new proteins, growth (DNA replication), and the regulation of transmembrane transport of ions and protons, and neurotransmitter levels (Table S8). Of particular interest is the CG hyper-methylation and, thus, potentially constitutive upregulation (Zhang *et al.*, 2006; Schmitz *et al.*, 2013; Yang *et al.*, 2014; Dubin *et al.*, 2015; Niederhuth & Schmitz, 2017) of genes involved in the catabolism of misfolded proteins (Table S8). Increased investment in the breakdown of heat-denatured proteins (Feder and Hofmann, 2002) may prime/adapt the affected shoots to future heat stress. This function was also hyper-methylated in control samples of highest performance and stress resilience (Table S11). However, parallel records of stress-responses in the methylome and transcriptome are necessary to link gene expression changes to changes in DNA methylation. Moreover, only removal of DNA methylation or the targeted change of methylation patterns, e.g. via CRISPR, will identify the relationship between heat-responsive methylation patterns and adaptive phenotypic changes.

Conclusions and Perspectives

Our study suggests that DNA methylation is functionally relevant for photosynthetic performance and heat stress resilience, and that a portion of the stress-responsive changes in CG methylation is sufficiently stable to allow for transgenerational plasticity in clonal lineages. In seagrass meadows composed of several genotypes, stress resilience, growth, and associated invertebrate species diversity is enhanced by genotypic variation (Hughes & Stachowicz, 2004; Reusch *et al.*, 2005; Ehlers *et al.*, 2008). In clonal meadows, epigenetic variation may play a similar role in the potential to secure function and resilience not only of *Z. marina* plants, but also of the entire associated ecosystem.

Due to anthropogenic stressors, nearly one-third of global seagrass area has disappeared over the last 100 years, and the rate of loss accelerated from ca. 1% yr^{-1} before 1940 to 7% yr^{-1} since 1990 (Waycott *et al.*, 2009). At the same time, rising temperatures open up new thermally suitable habitat in the Arctic (Krause-Jensen & Duarte, 2014). How fast and far warm-temperate and subarctic range edges will move polewards depends on the ability of seagrass to rapidly acclimate and adapt to rising temperatures and other environmental changes (Duarte *et al.*, 2018). Thus, future studies are needed to assess the adaptive value and transgenerational stability of the

epigenetic stress response, and to compare the ability to build up epigenetic variation between seagrass meadows of low and high clonal diversity (composed of a single or multiple clones), as well as between range center versus edge populations.

The functional role of methylation variation in plant genets is not only of fundamental interest but also of applied interest for management programs of clonal organisms designed to assess evolutionary potential and population stability, and to minimize the loss of biodiversity. Our results can be directly relevant for restoration of coastal biodiversity associated with seagrass ecosystems that largely depends on the success of replanted shoots to overcome natural variability (van Katwijk *et al.*, 2016). Given that 40% of all plant species can reproduce clonally (Tiffney BH, 1985), our findings are further important to other fields, such as invasion biology and crop breeding strategies (Bilichak & Kovalchuk, 2016) of clonal plants.

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Author Contribution

GH (project leader) and AJ were planning the project and designing the experiments. AJ, CB, IS, and GH collected the shoots. AJ analysed the data and wrote the manuscript. AJ, MK, AKSD, JAC, and IS performed the DNA extraction, library preparation, and sequencing. SAH, and YvdP were involved in data interpretation. All co-authors read and commented on the manuscript.

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Figures

Fig. 1: Baltic Sea sampling site of a > 1,000 years old clonal meadow where 42 *Zostera marina* shoots were collected along a 250 m transect.

Fig. 2: Methylation variation of transect (pre-acclimation) samples of *Zostera marina* exceeded the shift in methylation patterns in response to heat stress. The samples are plotted along the first two principle components (Dim) based on methylation profiles across all sequence contexts. Circles represent 95% confidence intervals around group means. Bracketed numbers represent the percentage of explained variation.

Fig. 3: Genetic relatedness did not restrict epigenetic differentiation. Bivariate methylation differences between were comparable within ramets of the same genet and between ramets of different genets of *Zostera marina* across all sequence contexts.

Fig. 4: Positive correlation between among-shoot difference in photosynthetic performance (PiAbs) prior to stress (a), and after recovery (b) with epigenetic distance prior to stress based on CG methylation patterns in gene regions (excluding transposable elements in genes) of *Zostera marina*; p: p-value, R: Pearson product-moment correlation coefficient.

Fig. 5: Positive correlation between among-shoot difference in photosynthetic performance (PiAbs) and epigenetic distance after recovery based on CHG methylation patterns in intergenic regions

(excluding transposable elements) (a) and transposable elements in intergenic regions (b) of *Zostera marina*; p: p-value, *R:* Pearson product-moment correlation coefficient.

Fig. 6: Methylation patterns in shoots of a *Zostera marina* clone changed in response to heat stress and did not return to pre-stress patterns after a 5-week recovery period. The samples are plotted along the first two principle components (Dim) based on methylation profiles across all sequence contexts. Circles represent 95% confidence intervals around group means. Bracketed numbers represent the percentage of explained variation.

Fig. 7: hyper-methylation dominates the stress response of *Zostera marina*. Stress-responsive methylation changes were memorized over the >5-week recovery period. TE: transposable element.

Supporting Information

Fig. S1: Photosynthetic performance (PiAbs, absolute values) for all heat-stressed shoots at control, stress, and after recovery.

Fig. S2: Proportion of methylated sites detected in genes, intergenic regions, and transposable elements (TEs), separated by CG and CHG sequence contexts.

Table S1: Seagrass samples that were taken along a ca. 250 m transect.

Table S2: Number of sequenced reads, trimmed (high-quality) reads, mapped, and uniquely mapped reads for each sample.

Table S3: Number of methylated CG and CHG sites in genes, intergenic regions, and transposable elements (TEs), listed for each transect sample.

Table S4: Microsatellite raw data. Microsatellite fragment lengths for 7 markers (2 columns = 2 alleles per marker) for each sample (ID in the first column).

Table S5: Mantel test results (p-values and Pearson's Correlation Coefficient R) for correlations between epigenetic and geographic distance at each sequence context.

Table S6: Photosynthetic performance measures. Mean values and standard errors of PiAbs for each of the 10 shoots at all three sampling points in the heat stress experiment.

Table S7: Number of differentially methylated sites between control vs. stress, control vs.

recovery, and stress vs. recovery samples listed for each sequence context.

Table S8: Enriched biological processes showing differential methylation in gene bodies between control and recovery samples.

Table S9: Mantel test results for correlations between epigenetic distance and performance differences at each sequence context. Correlations with Pearson's Correlation Coefficient $R > 0.69$ and $p < 0.05$ are highlighted in orange.

Table S10: Differentially methylated sites between two samples of highest and two samples of lowest photosynthetic performance.

Table S11: Enriched biological processes with increased methylation in control samples of high performance.

File S1: Adapters and primers used to prepare MethylRAD libraries.

File S2: Annotated reads-per-million for 42 transect samples.

File S3: Annotated reads-per-million for experimental samples (control, stress, and recovery).

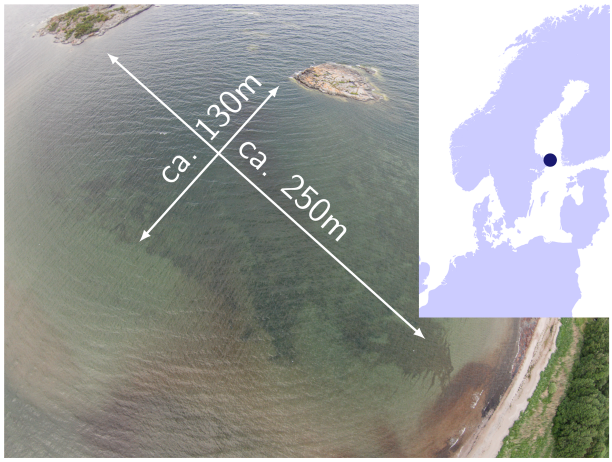

Figure 1

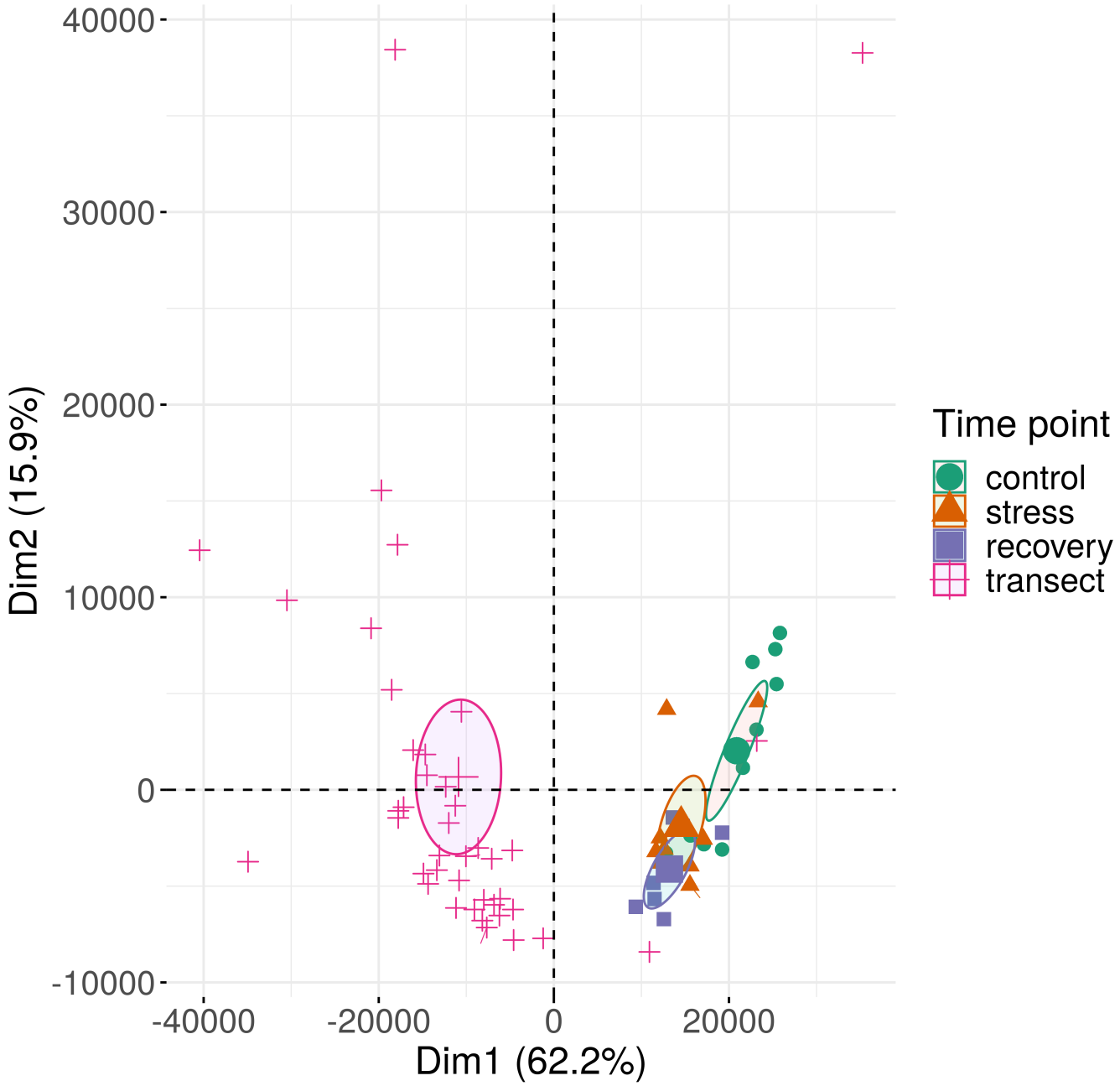
40000
30000
20000
10000
0
-10000
-40000
-20000
0
20000
Dim2 (15.9%)
Dim1 (62.2%)
Time point
control
stress
recovery
transect

Figure 2

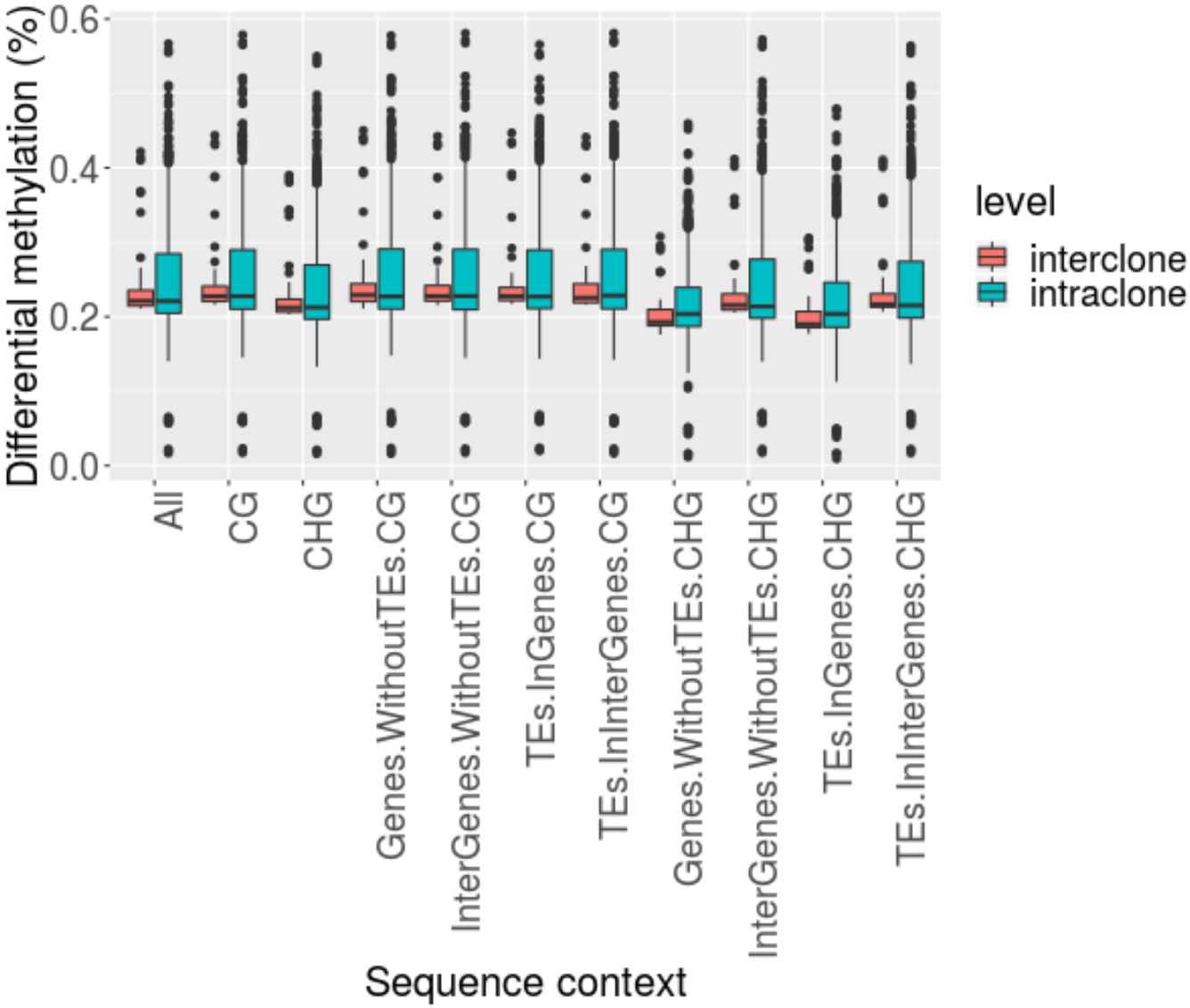
Differential methylation (%)
0.6
0.4
0.2
0.0
All
CG
CHG
Genes.WithoutTEs.CG
InterGenes.WithoutTEs.CG
TEs.InGenes.CG
TEs.InInterGenes.CG
Genes.WithoutTEs.CHG
InterGenes.WithoutTEs.CHG
TEs.InGenes.CHG
TEs.InInterGenes.CHG
Sequence context
level
interclone
intraclone

Figure 3

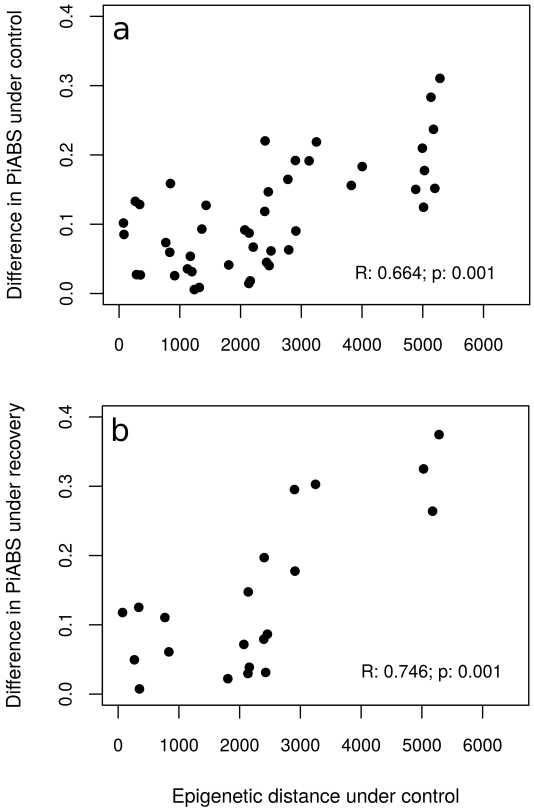
a
Difference in PiABS under control
0.0
0.1
0.2
0.3
0.4
R: 0.664; p: 0.001
0
1000
2000
3000
4000
5000
6000
b
Difference in PiABS under recovery
0.0
0.1
0.2
0.3
0.4
R: 0.746; p: 0.001
0
1000
2000
3000
4000
5000
6000
Epigenetic distance under control

Figure 4

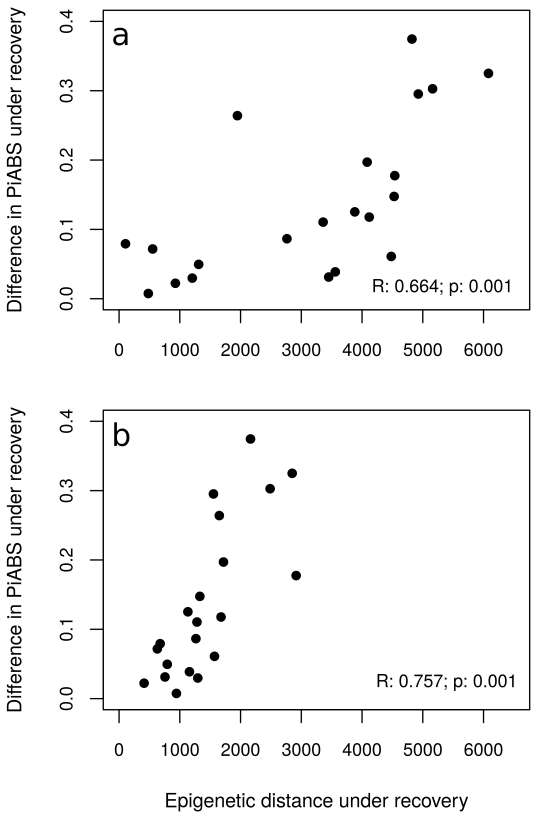
a
R: 0.664; p: 0.001
Difference in PiABS under recovery
0.0
0.1
0.2
0.3
0.4
0
1000
2000
3000
4000
5000
6000
b
R: 0.757; p: 0.001
Difference in PiABS under recovery
0.0
0.1
0.2
0.3
0.4
0
1000
2000
3000
4000
5000
6000
Epigenetic distance under recovery

Figure 5

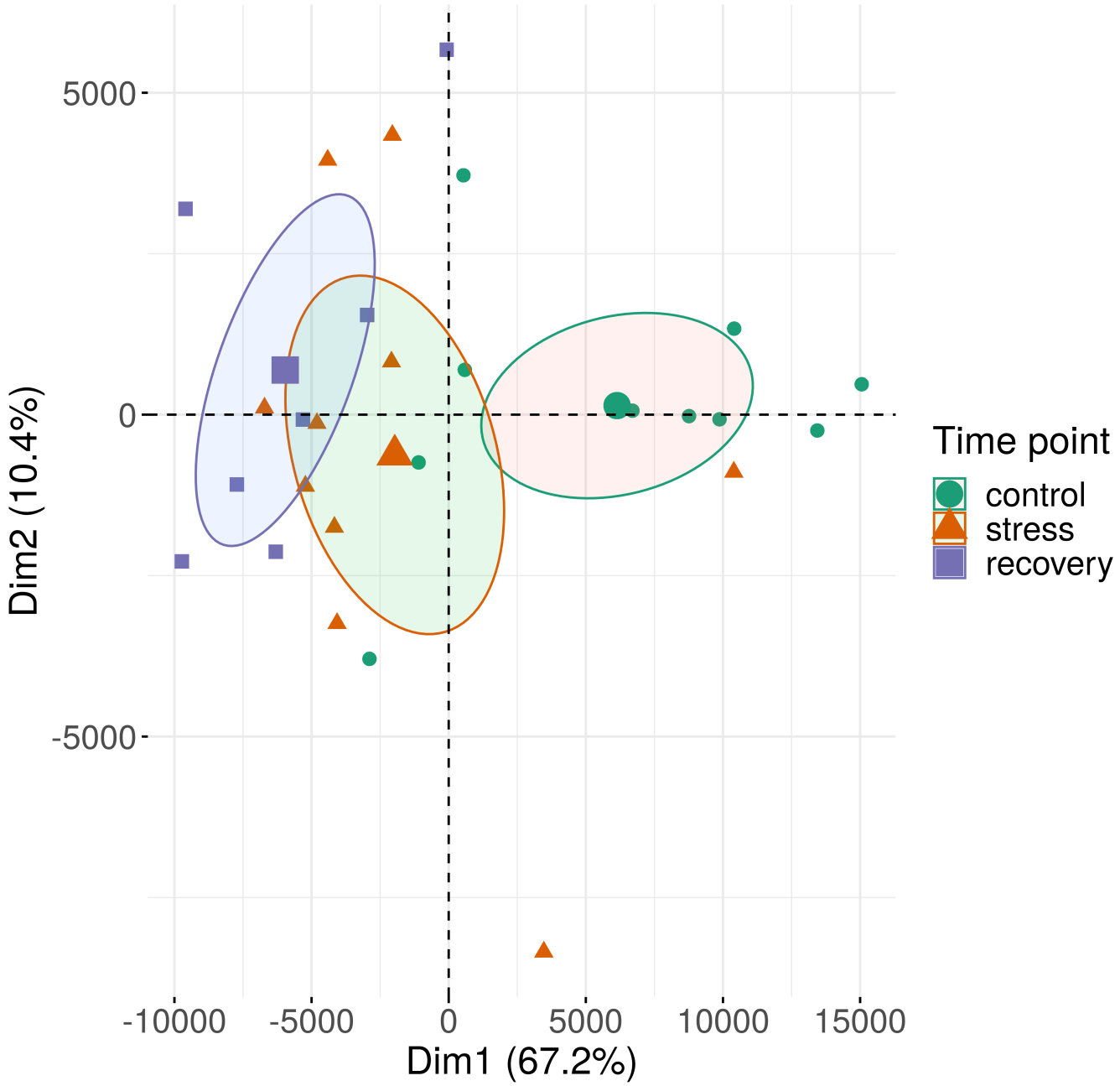
5000
0
-5000
Dim2 (10.4%)
-10000
-5000
0
5000
10000
15000
Dim1 (67.2%)
Time point
control
stress
recovery

Figure 6

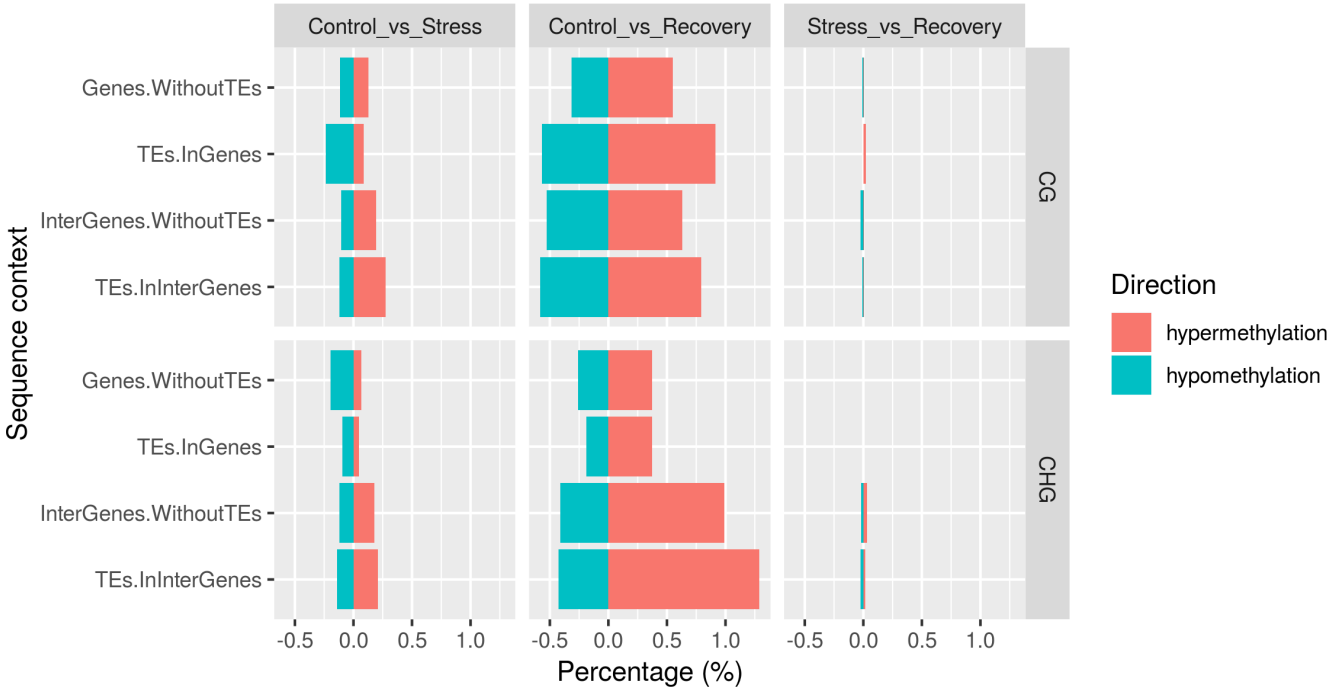
Control_vs_Stress
Control_vs_Recovery
Stress_vs_Recovery
CG
CHG
Sequence context
Genes.WithoutTEs
TEs.InGenes
InterGenes.WithoutTEs
TEs.InInterGenes
-0.5
0.0
0.5
1.0
Percentage (%)
Direction
hypermethylation
hypomethylation

Figure 7