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The methylome of clonal seagrass shoots shows age-associated variation and differentiation of roots from other tissues

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ABSTRACT

Factors influencing variance of DNA methylation in vegetatively reproducing plants, both terrestrial plants and aquatic seagrasses, is just beginning to be understood. Improving our knowledge of these mechanisms will increase understanding of transgenerational epigenetics in plant clones, of the relationship between DNA methylation and seagrass development, and of the drivers of epigenetic variation, which may underly acclimation in clonally reproducing plants. Here, we sampled leaves, rhizomes and roots of three physically and spatially separated ramet sections from a clonally propagated field of the seagrass *Zostera marina*. Using reduced methylome sequencing, we studied variations in the methylome of seagrass *Zostera marina* between the sampled tissue types and across age groups. Our analysis of ramets of different ages showed variations in methylation between older and younger samples in both specific methylation patterns and global methylation levels. Our analysis of tissue types showed a marked differentiation of the roots from the rhizomes and leaves, which showed more similar methylation patterns. These findings are in agreement with the strong connection of DNA methylation and plant development and tissue differentiation. We also suggest an effect of differential environmental exposures on the methylome of the younger versus the older ramets due to the forming of molecular stress memories.

1. Introduction

Epigenetic mechanisms regulate gene expression without altering the DNA sequence itself [1]. DNA methylation, along with other epigenetic mechanisms such as histone modifications and non-coding RNA (ncRNA), play an important role in the regulation of gene expression, genome stability, transposon silencing and chromosome interactions and therefore also regulates development and differentiation of cells and tissues [2,3,4]. DNA methylation is often associated with inhibition of gene expression, but in some cases, it can rather increase gene expression [5,6].

In terrestrial plants, DNA methylation occurs at the C5 position of a cytosine at a CG, CHG or CHH sequence context where H represents A, C, or T, and the methylation is initiated and maintained by DNA

methyltransferases [7,8]. DNA methylation regulates gene expression which relates to cell functions, and the methylation pattern varies between plant organs and tissues such as roots, leaves and reproductive tissues [9,8]. DNA methylation is dynamic throughout the plant's development, and affects the development of plant tissues and cell differentiation [7,3]. And finally, differential gene expression by DNA methylation affects biological processes and specific functions such as photosynthesis, nutrient transportation, or storage [10,3,4]. Thus, DNA methylation plays an important role in plant development and in regulating plant cell functions in different cell and tissue types.

Methylation patterns of terrestrial plants generally changes with age, and differs between tissues at different stages of maturation due to the role of DNA methylation in regulating the developmental process [8]. The patterns can be used to determine age within the same plant or in

Abbreviations: BP, biological process; CC, cellular component; GO, gene ontology; MF, molecular function; PCA, principal component analysis; PSU, practical salinity unit; TE, transposable element.

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vegetatively reproducing organisms [11,12]. DNA methylation also plays an important role in plant stress response and can be altered by environmental stressors [10,13]. Changes that are induced by stress may be involved in forming a stress memory that enables the organism to respond faster and/or stronger to future exposures to stress, especially when the stress occurs at a young life stage [14]. Thus, the methylation pattern of a mature organism is affected by environmental factors experienced at a younger stage, leading to differences in methylation patterns between plants previously exposed to different environments [15].

Although resetting DNA methylation can occur under sexual reproduction its status is partially heritable in plants, as variations in heritable methylation changes known as epialleles can occur when DNA methylation is not completely reset during reproduction [8,16]. The DNA methylation can be altered by environmental factors such as temperature, light and nutrient availability, and such alterations can in some cases be transferred to the next generation [17,13], in particular under clonal reproduction. For example, in clonally reproducing duckweed, CG and CHG methylation variations induced by temperature can be stably transferred across several generations [18,19]. In summary, DNA methylation undergoes dynamic changes under development and in response to environmental factors and is inherited to a varying degree but with more stable transgenerational transfer via clonal reproduction.

Eelgrass *Zostera marina* is a seagrass species widely distributed in the northern hemisphere, which forms seagrass meadows that are highly productive and diverse ecosystems. Through slowing water flow, stabilizing sediments and thus altering the shape of their environment, seagrasses act as foundation species and form habitats essential for fish and invertebrates, protect against coastal erosion, maintain water quality, and provide carbon and nutrient sequestration services [20]. Seagrass ecosystems, however, are threatened by anthropogenic stressors such as increased sea surface temperatures and marine heatwaves, rising sea levels, and sediment and nutrient and chemical pollution from agricultural runoff [21,22]. In the Baltic Sea, *Z. marina* meadows are predicted to be highly threatened under future climate conditions, and their loss would have severe impacts on ecosystem services [23].

Z. marina can reproduce both sexually through flowering, and vegetatively by extension of its rhizomes. Therefore, the seagrass can form clonal meadows, some of which consist of a single large clone or a small number of clones, and these clonal seagrass meadows lack the genetic variability observed in sexually reproducing meadows [24,25]. Seagrass populations at the coasts of the Baltic Sea show particularly low levels of genetic diversity, possibly caused by environmental factors favoring clonal over sexual reproduction, and by past losses of seagrass meadows [26,27]. However, variation in DNA methylation can still be high in clonal meadows, and is associated with functional variance in *Zostera marina*, for example it is relevant to photosynthetic performance [28]. Diversity of DNA methylation and its contribution to phenotypic diversity has been observed in several clonally reproducing terrestrial plants, for example white poplar *Populus alba* L., saffron crocus *Crocus sativus* L., and white clover *Trifolium repens*, [29,30,31]. Higher epigenetic diversity in plant population is associated with higher biomass production compared with epigenetically uniform variants, and transgenerational epigenetic variations has been proposed as a mechanism that aids adaptation of clonally reproducing plants to climate change [32,33]. Therefore, the epigenetic variability of vegetatively propagating organisms is a topic of great interest for conservation purposes.

The causes of methylome variations are complex and could be an interplay of developmental processes, stochastic changes and environmental effects [13,34,35], consequently many aspects of the background and functionality of seagrass DNA methylation remain unknown. In this study, we characterize how DNA methylation varies between different tissue types of *Z. marina* (root, leaf, and rhizome) and how variation may relate to functional differences. We also identify differences in DNA methylation between clones of different ages within the same clonal

meadow to explore whether the methylome is more affected by trans-generational effects or effects related to different stages of growth and development.

2. Materials and methods

2.1. Sampling

Zostera marina samples were collected from a clonal seagrass in Hinderbengtsteviken, Åland Islands, Finland (60°09'50.4"N, 19°31'48.1"E) in June 2015. Samples of the seagrass were obtained at 39 m (section 1), 87 m (section 2), and 141 m (section 3) along a 250 m transect from −0.5 m to −3 m mean sea level. Shoots were transported in fine mesh bags in cooling boxes with seawater to the field station at Nord University, Bodø, Norway, and replanted in plastic containers (10 × 15 cm) in sand originating from the sampling site. Replanted shoots were kept for 8 wks in 5.5 PSU water (corresponding to sampling site salinity, mixed from freshwater and 32PSU natural filtered seawater) at a 40 cm water level in a 1280-L aquarium at 15 °C (field temperature) at a 16:8 h light–dark cycle under light-saturating conditions (200–220 μmol m^{−2} s^{−1}, OSRAM Fluora, 150 W). Airstones provided constant water motion and water was renewed by 50 % once a week after removing epiphytic algae. The innermost leaf of the biggest shoots was flash-frozen for DNA extraction, insuring methylation patterns of the shoot samples would reflect the youngest leaves. All leaves were removed from the younger shoots. Samples were also taken from rhizomes and roots. The positions of the samples in relation to each other are illustrated in Fig. 1. For the division into age groups (Fig. 1), the “young” shoots are located at the end of each ramet, and the “old” shoots are at least 5 steps away from the “young”. The samples between the young and old group are categorized as a “medium” age group, that was omitted from further analysis because of the low sample size and high variation of methylation patterns which did not characterize it as a distinguished group in the PCA (4 “medium” age samples). Regarding the sample sizes, for the age groups analyses we included 12 samples in the “old” age group and 6 samples in the “young” group. For the tissue type analyses, we included 2 root, 10 leaf, and 10 rhizome samples.

2.2. DNA extraction and sequencing

DNA was extracted and sequencing libraries were prepared in accordance with the MethylRAD protocol [36], with modifications described in Jueterbock et al. [28]. MethylRAD is based on the methylation-dependent restriction enzyme *FspEI* and targets about one-eighth of fully methylated CCGG and CCWGG sites, thereby capturing methylation patterns in CG and CHG sequence contexts [36]. Quality trimming and mapping to predicted MethylRAD tags from the in silico digested *Z. marina* genome v2.1 from ORCAE [37] are described in Jueterbock et al. [28]. The resulting methylation sites were annotated with the v2.1 gff3 file from ORCAE [37], and sorted to genes, intergenic regions, TEs, and CG and CHG context methylation.

2.3. Methylation levels

We investigated if methylation levels differed between tissue types and different connected ramet sections (1, 2 and 3). Average methylation levels as reads per million (RPM) and number of methylated sites was calculated separately for each section, and separately for the different tissue types: root, rhizome and leaf. Reads per million were calculated as

$$\frac{\text{reads per gene} \times 10^6}{\text{reads per sample library}}$$
 We used the non-parametric Wilcoxon rank sum tests to test for differences in mean methylation levels. For the rhizomes and leaves, we also compared the methylation levels and numbers of methylated sites for the different age groups using the same method. In addition, the average methylation level for each position throughout the

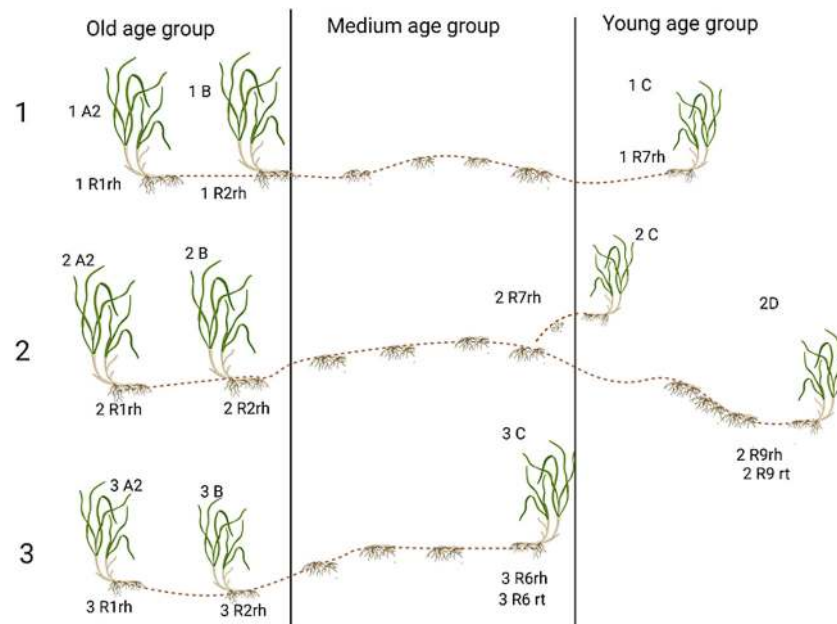


Fig. 1. Structure of three sampled ramet sections 1,2,3 growing from left to right. Leaf samples are marked with letters A-D, rhizome samples are marked “rh” and root samples are marked “rt”. The horizontal lines illustrate how plants on the three ramet sections 1, 2 and 3 are connected. The direction of growth is left to right on the figure. Numbering starts at the oldest sampled shoot and is an indication of stepwise spatial distance between shoots. Created with [BioRender.com](https://www.biorender.com).

genes and 10 kb flanking upstream and downstream of transcription start and end sites were calculated, producing curves for methylation levels throughout the genes.

2.4. PCA and pairwise epigenetic distances

We used the R packages FactomineR and factoextra [38,39] for PCA (principal component analysis), with the methylation data normalized to RPM. In order to test for changes in methylation in the series of shoots along the same ramet section, samples were grouped by age groups in the PCA plot. Ellipses in the PCA plots were drawn as “confidence” type ellipses, which represent 95 % confidence intervals for the means. In an additional PCA plot, samples were grouped by the tissue types “leaves”, “root” and “rhizome” to investigate differences in methylation patterns between tissue types.

Epigenetic distances among the samples were estimated as the Euclidean distances on the two-dimensional PCA plot using the ‘dist’ function of the R package “stats”. We tested for significant differences in epigenetic distances between samples of old and young age groups (as described above), and between within and the section 1, 2 and 3 using Wilcoxon rank sum test as the data did not fit assumptions of normality for *t*-tests. Comparisons were made separately for CG and CHG context methylation, and for this purpose new PCA analyses, which provided the Euclidean distances, were done separately for CG and CHG context methylation sites.

To investigate whether the methylation pattern changes in the clonal shoots along the sections, we tested for Spearman’s correlation between epigenetic distance and the number of shoots separating the ramets (a proxy for age difference). Separate tests were performed for each tissue type, for the different methylation contexts CG and CHG, and for the 3 different sections. *P* values were adjusted with the Benjamini-Hochberg procedure to control for false discovery rate [40]. We also conducted Mantel tests with the non-parametric “spearman” option using the R package “vegan” to test for correlation among the epigenetic distance and the distance between samples along the ramets. The *p* values were adjusted with the Benjamini-Hochberg method [40]. For these tests, the root samples were excluded because of their low sample size (2) and their great distance from the other tissue types.

2.5. Differential methylation and GO enrichment analyses

To investigate which sites were differentially methylated between tissue types and between age groups, we performed differential methylation analysis using the “DESeq2” R package, which is robust to unequal sample sizes [41]. With DESeq2, statistical significance at the low sample size of 2 (roots) can be expected to be real and rather an underestimation of what we could expect at a higher sample size. We considered sites with Benjamini-Hochberg adjusted *p* value <0.05 to be differentially methylated.

To aid the biological interpretation of the results from the differential methylation analyses, the output lists of differentially methylated genes were analysed for GO term enrichment with Fisher’s exact test for enrichment of gene ontology using the R package “topGO” [42] with GO terms from the v2.1 *Z. marina* genome annotation from the ORCAE database [37]. The *p* values of the enriched GO terms were then adjusted with Benjamini-Hochberg *p* value correction. To reduce redundancy and summarize the GO enrichment, the enriched GO terms were reduced by eliminating similar terms with the R package “rrvgo” [43], which applies a similarity matrix where the similarity of the GO terms is calculated, and one term is selected as the representative for a group of similar terms.

3. Results

3.1. Variation in methylation levels among tissue and age groups

The methylation levels show significant differences between tissue types according to the Wilcoxon Rank Sum tests with Benjamini-Hochberg *p*-value correction (Fig. 2). The only trend that is consistent among 2 ramet sections is that CHG methylation levels were highest in roots (Fig. 2). The leaves had higher CH and CHG methylation levels compared with rhizomes, except in section 2. There are no significant differences between the numbers of methylated sites for tissue types or age groups (Supplementary Figs. 2 & 3). The young age group had the highest methylation levels in rhizomes and the old age group had highest methylation levels in leaves (Fig. 3). In roots and rhizomes, average CG methylation levels over all genes dipped around the

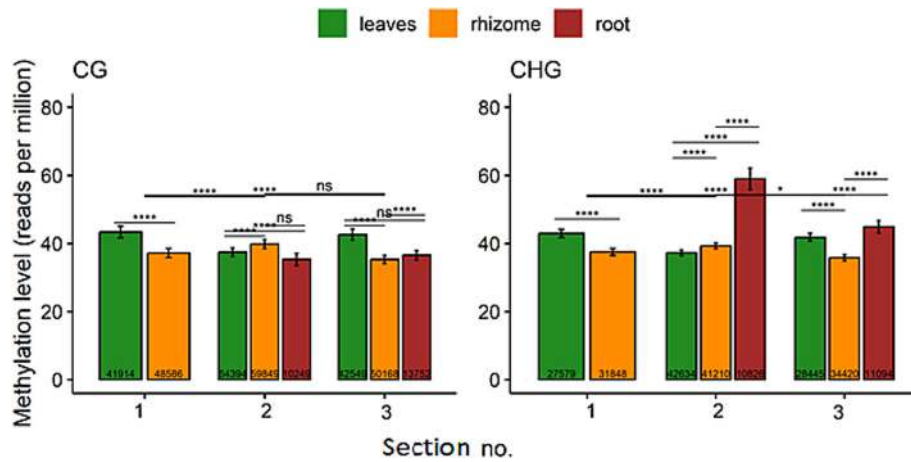


Fig. 2. The average methylation levels at CH and CHG sites as reads per million for the leaves, roots and rhizomes of the three studied ramet sections (number 1, 2 and 3). Significance codes after *p*-value correction (Benjamini-Hochberg) for multiple comparisons: ' < 0.05 ': *, ' < 0.01 ': **, ' < 0.001 ': ***, ' < 0.0001 ': ****. Numbers at the bottom of the bars indicate numbers of methylated sites.

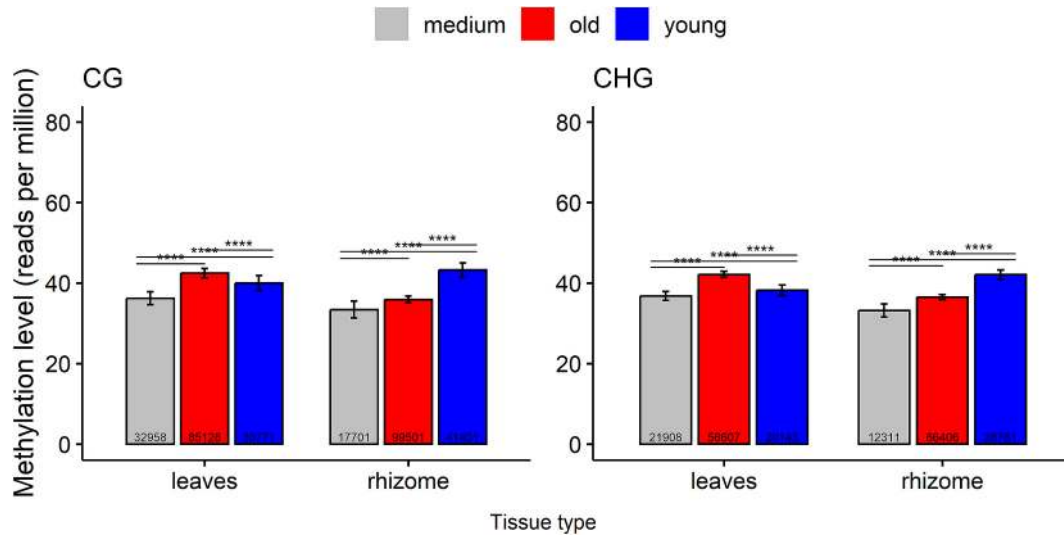


Fig. 3. The average methylation levels at CH and CHG sites as reads per million for the leaves and rhizomes of the three studied sections, comparing the different age groups young, medium and old. Significance codes after *p*-value correction (Benjamini-Hochberg) for multiple comparisons: ' < 0.05 ': *, ' < 0.01 ': **, ' < 0.001 ': ***, ' < 0.0001 ': ****. Numbers at the bottom of the bars indicate numbers of methylated sites.

transcription start and end sites, while CHG methylation dipped only near the start sites (Fig. 4). As for the roots, the methylation patterns deviated from that of the other tissues (Fig. 4).

3.2. Overall methylation patterns in tissue- and age-specific groups

Overall, PCA of RPM normalized methylation levels revealed tissue- and age-specific grouping. The overlap of 95 % confidence ellipses in the PCA plot indicates that overall methylation patterns differed between the roots and other tissue types, but not between leaves and rhizomes (Fig. 5). Similarly indicated by the 95 % confidence ellipses, the patterns for age groups were dependent on the distance between older and younger leaves/rhizomes (Fig. 5A). The medium age group is spread between the other groups and omitted from further analysis. As the first two principal components (Fig. 5A & 5B) explained 88.9 % of the total variance in DNA methylation patterns, most of the overall variation is dependent on these factors.

3.3. Differential methylation between groups and GO term enrichment

Comparisons of "DESeq2" results for roots with other tissue types showed distinctly more differentially methylated sites than the comparison between leaves and rhizomes (Table 1; complete list of differentially methylated sites: Supplementary tables 3–5) and is consistent with the spacing of the ellipses in the PCA plots (Fig. 5A). The young and old age groups, which formed different groups in the PCA (Fig. 5B) had 66 differentially methylated sites (Table 1, Complete list: Supplementary materials 2).

Comparisons of "DESeq2" results between tissue types roots vs. leaves and roots vs. rhizomes show differential methylation in both flanking and gene regions, including in repeats within the gene regions (Fig. 6, Fig. 7). Both comparisons, roots vs. rhizomes and root vs. leaves had overlapping enriched GO terms (e.g., GO:0065007 biological regulation, GO:0000103 sulfate assimilation, GO:0017009 protein-phycocyanobilin linkage, GO:0031297 replication fork processing) as well as unique (e.g., GO:0051274 beta-glucan biosynthetic process in rhizome, GO:0051273 beta-glucan metabolic process in leaves). No GO terms were enriched in the leaves vs. rhizomes comparison. The age

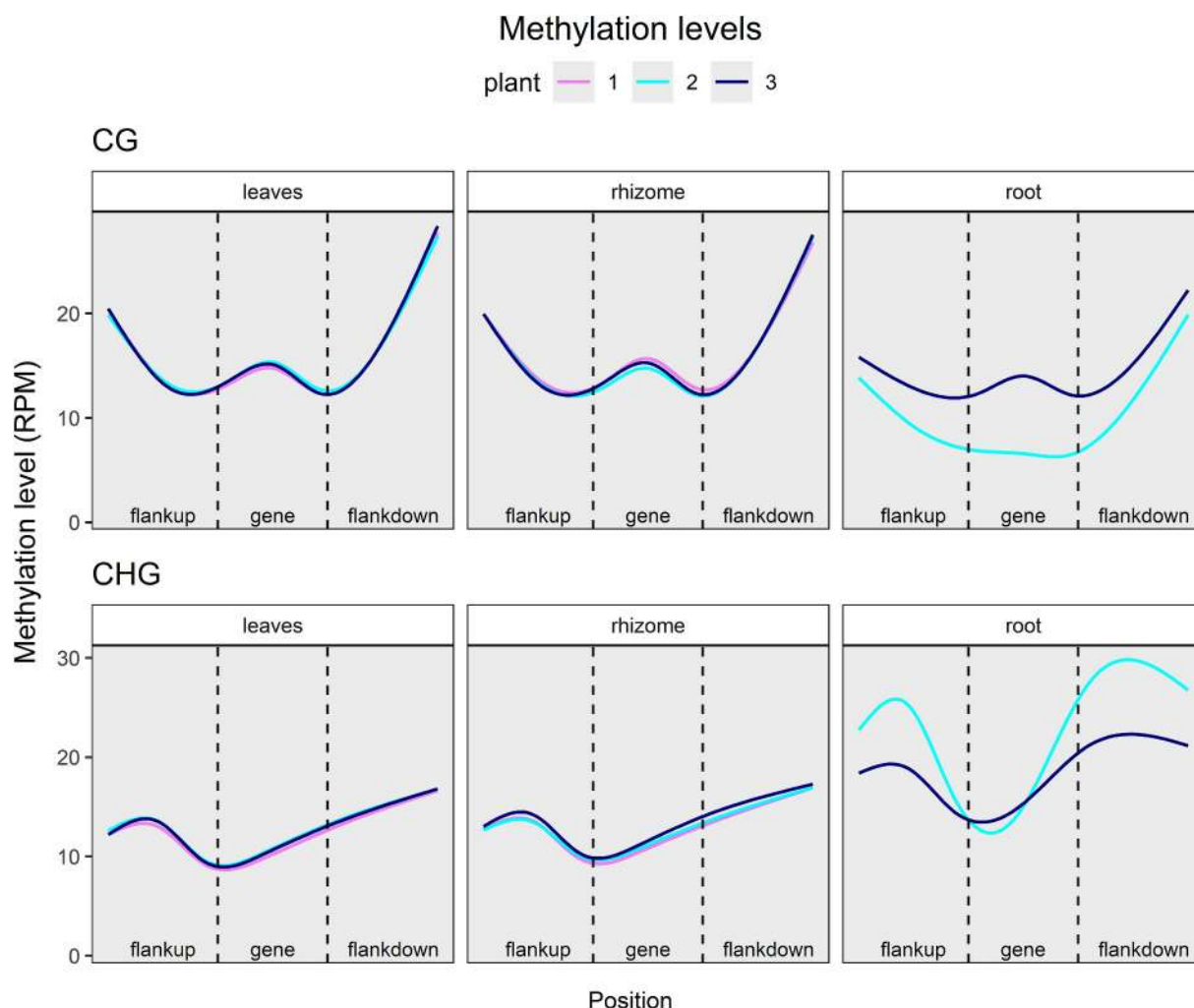


Fig. 4. Averaged methylation level throughout genes in CG and CHG contexts, normalized as read per million. The dotted lines represent the start and end points of transcription, while the flankup and flankdown sections continue for 10 kb upstream and downstream.

groups comparison finds enriched GO terms only in repeats (TEs) in non-genic regions (Fig. 8), which are assigned GO terms because of their proximity to genes. The biological processes that these genes are involved with connected to regulation of transcription (GO:0045893), chromosome organization (GO:0051276), double-strand break repair (GO:0000724, GO:0006302), and protein ubiquitination (GO:0016567), suggesting differential regulation of genes relating to these processes between age groups.

3.4. Correlations of epigenetic and spatial distances

The correlation between the epigenetic distance between samples and the distance between samples is typically positive or near zero in the Spearman's correlation and Mantel tests (Table 2, Supplementary Table 1, Fig. 9), with the correlation being significant for only CHG and CG context methylation for only section 2 (Table 2) according to the Benjamini-Hochberg adjusted p values. In the other sections, correlation is near zero (Table 2). For the Mantel tests, no correlations were significant after adjusting the p values with the Benjamini-Hochberg method (Supplementary Table 1). This indicates that epigenetic distance and spatial distance in the field are not directly correlated.

3.5. Pairwise epigenetic distances in age groups

Our Wilcoxon rank sum test showed no significant differences in

mean pairwise Euclidean distance between samples of the same section compared to samples from two different sections (distances: Supplementary Fig. 3), indicating that the epigenetic variance was no greater among than within each ramet section. For CG context methylation the p value of the Wilcoxon rank sum test was 0.40 while for CHG context methylation the p value was 0.31. The Wilcoxon rank sum test for pairwise distances within groups between the young and old age groups (Fig. 10) shows no significant difference between the groups with the p value 20 for CHG and 0.23 for CG context methylation, indicating that the epigenetic distances between samples are similar within the young and old age groups.

4. Discussion

Methylated sites in the *Zostera marina* methylome differed between tissue types (roots vs. leaves and roots vs. rhizomes) and between age groups. This is not surprising, given the role of DNA methylation in plant development and tissue differentiation [5,13]. While phenotypic variation among tissue types is obvious, our findings illustrate how age differences provide a measure of epigenetic, and thus potentially phenotypic, variety in the vegetatively propagated seagrass meadow.

Our data show that rhizomes possess a methylation pattern similar to leaves but not roots, suggesting that the rhizomes, despite of growing underground, resemble in molecular function rather the leaves than the roots. In addition to the specific differentially methylated sites, our

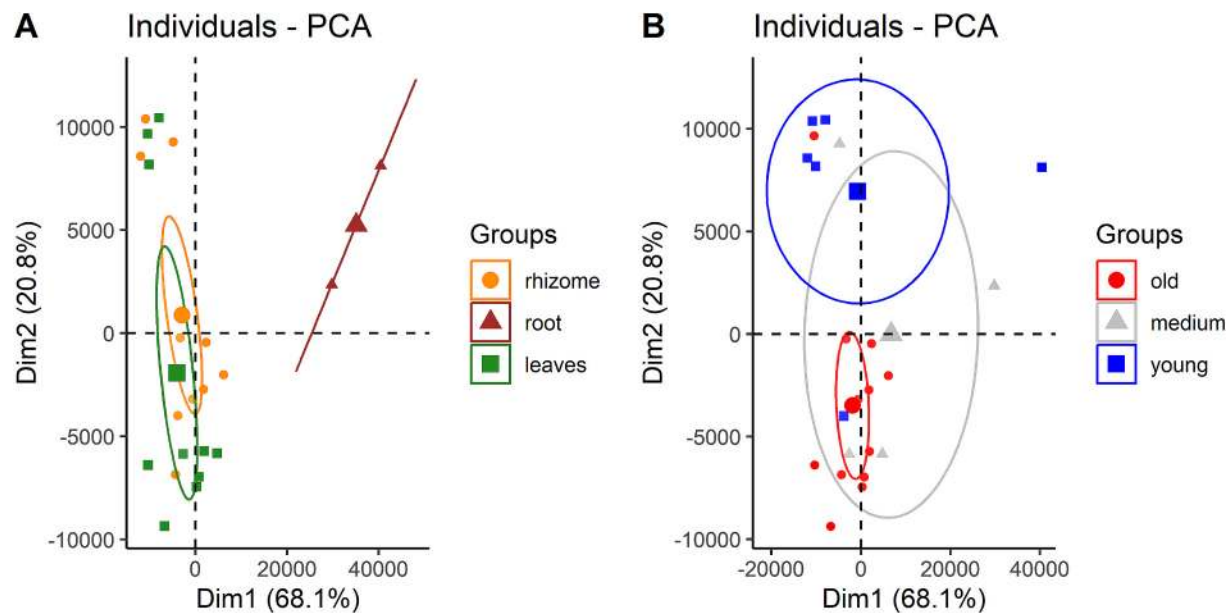


Fig. 5. A: Methylation patterns in samples based on principal component analysis with tissue-specific grouping. B: Methylation patterns in samples based on principal component analysis with age grouping (see Fig. 1). Samples of all tissue groups are included. The larger points represent the mean of each group, the ellipses show the 95 % confidence intervals around the group means.

Table 1
Numbers of differentially methylated sites for tissue type and age group comparisons, both as total numbers and split by CG/CHG methylation context and location of the methylation sites (Flanking/Gene/Repeats in gene/Repeats (TE) outside of gene /Other). The “Flanking” category represents 10 kb distances upstream/downstream from the transcription start or end sites. Sites were counted as differentially methylated when adj. *p* value <0.05.

Comparison	Differentially methylated sites (adj. <i>p</i> > 0.05)	CG	Location of CG methylated sites		CHG	Location of CHG methylated sites	
Roots/rhizomes	11,005	6397	Flanking	1513	4658	Flanking	322
			Gene	213		Gene	61
			Repeats, genic	747		Repeats, genic	515
			Repeats, nongenic	3808		Repeats, nongenic	3673
			Other	116		Other	77
Roots/leaves	9844	5699	Flanking	1358	4145	Flanking	301
			Gene	193		Gene	55
			Repeats, genic	662		Repeats, genic	456
			Repeats, nongenic	3380		Repeats, nongenic	3262
			Other	106		Other	71
Leaves/rhizomes	18	13	Flanking	6	5	Repeats, genic	1
			Repeats, genic	7		Repeats, nongenic	3
						Other	1
Old/young age group	66	36	Flanking	1	30	Repeats, genic	2
			Repeats, genic	3		Repeats, nongenic	28
			Repeats, nongenic	32			

samples showed higher levels of CHG methylation in the roots (Fig. 2). In other plants, elevated DNA methylation levels in roots following salt exposure has been observed, and were especially pronounced in salt-tolerant olive cultivars [44,45]. Findings from terrestrial plant studies point to gene body methylation influencing differential gene expression between plant structures, such as roots, young leaves and mature leaves [9,46,47]. For example, in the model grass species *Brachypodium distachyon*, DNA methylation varied between the leaves and floral buds in CG, CHG and CHH contexts and the differential gene body CG methylation covaried negatively with gene expression [46]. In *Catharanthus roseus*, DNA methylation and gene expression covaried between tissue types with most variation in gene body CG methylation, and a low negative correlation between DNA methylation and gene expression [9]. In our study, roots versus leaves/rhizomes showed differential methylation within the gene body as well as regulatory sequences, with more differential methylation at CG than CHG sites (Figs. 6–7 & Table 1), which may be involved in the differentiation of these tissue types. While DNA methylation is often associated with reduced gene expression, the

correlation between DNA methylation and gene expression varies among plant species, methylation context (CG, CHH, CHG), and between the gene body, regulatory regions and TEs [48,46]. Thus, further studies of DNA methylation and its covariation with transcriptomics would be needed to determine the functional effects of the differences in methylation pattern between roots and leaves/rhizomes observed in this study.

While our study lacks transcriptomic data, one connection between gene function and the observed differential methylation is related to sulphate accumulation in roots. The GO term sulphate assimilation (GO:0000103) shows significantly higher at gene flanking sequences methylation in the leaves and rhizomes compared to roots (Figs. 6 & 7). Sulphate related functions in the root are especially important in sea-grass which, in order to survive in sulphide-rich sediments, detoxify sulphide from the sediment by oxidation to sulphate (along with other detoxification methods), which can then be incorporated in the plant [49]. Processes related to the oxidization of sulphide to sulphate in an oxygenated area around the roots and following accumulation of



Fig. 6. Reduced GO enrichment terms as biological process, cellular component and molecular function for the comparison between roots and rhizomes. The arrows indicate in which tissue type the term is more highly methylated.



Fig. 7. Reduced GO enrichment terms as biological process, cellular component and molecular function for the comparison between leaves and rhizomes. The arrows indicate in which tissue type the term is more highly methylated.

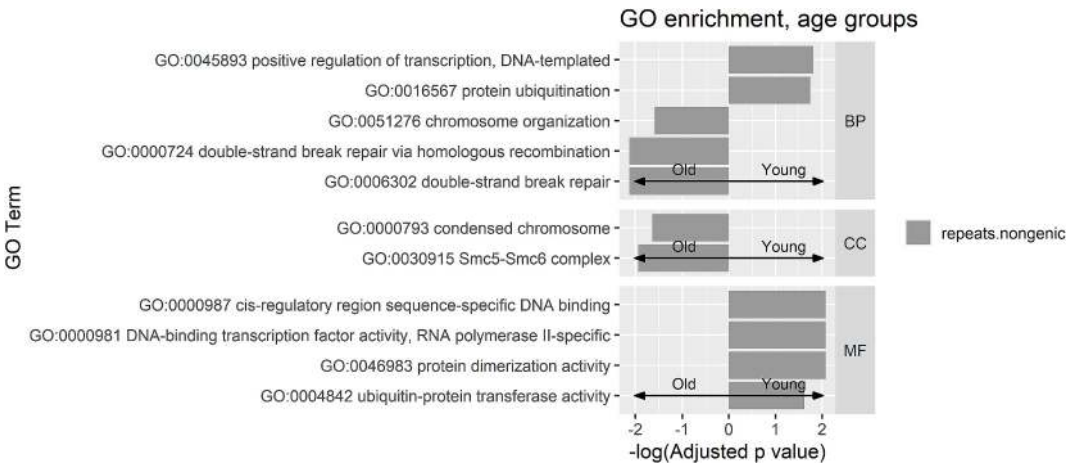


Fig. 8. Reduced GO enrichment terms as biological process, cellular component and molecular function for the comparison between the old and young age group. The arrows indicate in which group the term is more highly methylated.

Table 2
Results of Spearman's correlation tests for the correlation between epigenetic (Euclidean) distance and the distance between samples as number of shoots separating the ramets. P values are adjusted with the Benjamini-Hochberg method.

Section	Context	Spearman's correlation	P	Adj. P
1	CHG	−0.10687	0.704633	0.971087
2	CHG	0.456117	0.014707	0.044121
3	CHG	0.00737	0.979203	0.979203
1	CG	0.068173	0.809239	0.971087
2	CG	0.553659	0.00224	0.013439
3	CG	−0.28191	0.308708	0.617416

sulphate in the plant may explain lower methylation and, thus, potentially increased expression of genes related to this function in the root [49,50]. Sulphur also accumulates in seagrass rhizomes [49], however the methylation of this GO term in the rhizome was more similar to leaves than to roots. Hence, a connection between the enriched methylation of sulphur-related genes and sulphide detoxification processes needs further confirmation.

Methylation patterns along gene bodies in *Z. marina* are similar to terrestrial plants, but some differences exist, especially in the root tissue

[51]. Two root samples displayed unique patterns in methylation along gene regions, supporting their epigenetic difference to the other tissue types. Among the genes of leaves and rhizomes, CG methylation levels tended to peak in the central region of the genes and in flankup/promoter and flankdown regions, and lower near the start- and endpoints of the gene body (Fig. 3) in agreement with studies on other plants such as *Arabidopsis thaliana*, soybean *Glycine max*, and pigeon pea *Cajanus cajan* [52,53]. CHG methylation levels decreased near the TSS, in agreement with previous research on other plants [51,52], but did not show a distinct peak in the central region of genes, more in agreement with data on pigeon pea [52]. However, ramets appear to be more variable in methylation patterns of their roots, which both lacked an increased level of CG methylation in gene bodies.

Methylation patterns indicated by Euclidean distance in PCAs changed relatively rapidly along the ramets from the older to younger clones (Fig. 10), indicating either 1) the methylome experiences too high levels of epimutations to be reliably inherited across clonal generations, or 2) the methylome mostly reflect developmental/functional differences related to age but is rather stable among shoots of the same age group. That functional differences related to age groups better explain among-ramet differences compared to location in the meadow or physical distance among shoots, may be partly explained by the

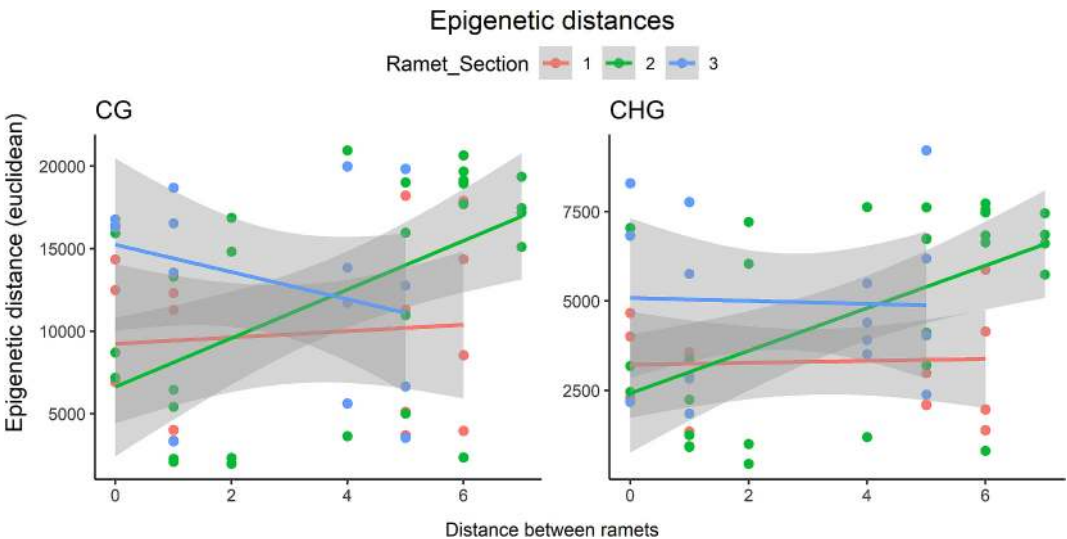


Fig. 9. The distance as number of ramets between each sample plotted against the epigenetic distance between the samples, with regression lines and standard errors.

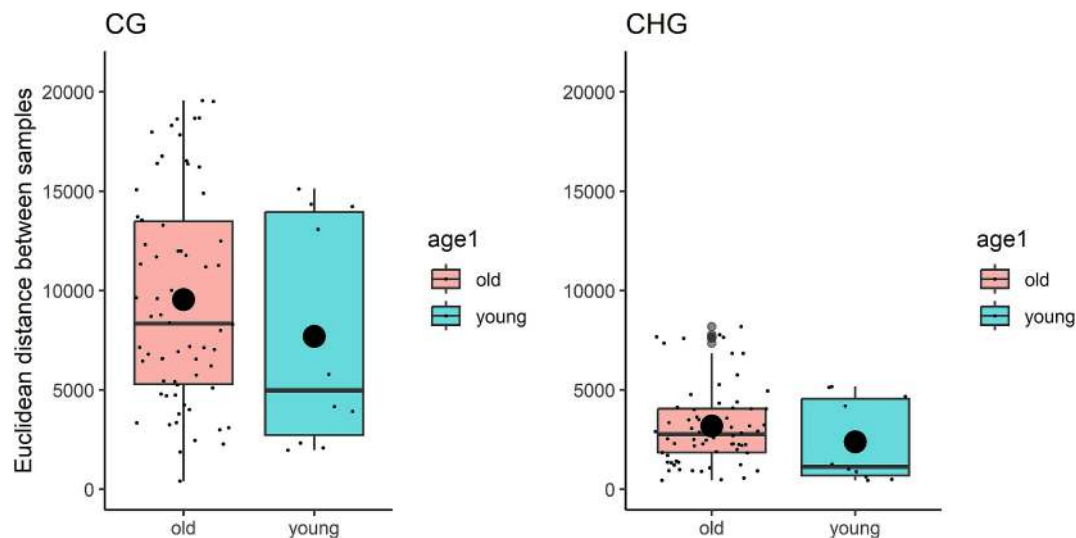


Fig. 10. The Euclidean distance (representing epigenetic distance) between samples (pairwise) of the young and old age groups, for CG (left) and CHG (right) methylation contexts. The large points represent the means.

predominance of mitotic over meiotic cell divisions in clonal seagrass meadows that allows for a stable transfer throughout clonal propagation [54]. Accordingly, plant age in pine also explained variation in CG and CHG context DNA methylation, mostly outside of annotated genes, over a longer time span of ca. 119 years [12]. An explanation for differential methylation between age groups may be that DNA methylation has an important role in controlling plant growth and development. For example, in maize GC and CHG gene body methylation differs among leaf zones that are at different stages of maturation [55,4]. Different stages of growth and development between the age groups could therefore explain the differential methylation among ramets of the same genet.

Epigenetic distance between shoots did not correlate with physical distance in a previous study of *Z. marina* [28], in agreement with our data. Thus, differential methylation in a clonal seagrass meadow is more affected by age of ramets rather than spatial distance. In the seagrass *Posidonia oceanica*, DNA methylation varied with leaf tissue age both within and among leaves, suggesting that epigenetic changes occur during their development and could affect leaf maturation [56]. Older plants, which in this study differed epigenetically from younger plants (Fig. 5, Fig. 8) may have had the chance to experience a wider range of environmental conditions, and, thus, to form molecular stress memories that differentiate them from younger plants but also from each other, as various abiotic stressors could have led to DNA methylation responses [10]. In the seagrass species *Z. marina* and *P. oceanica*, the changes in DNA methylation levels or patterns have occurred after heat stress exposure [57,28]. In addition, the methylome of *P. oceanica* responded to eutrophication nutrient stress [58]. These stress responses hold the potential for a molecular stress memory in the seagrasses, which may in part explain the methylation differences between older and younger individuals in our study. Thus, the relation between epigenetic distance and ability to recover from heat-stress [28] could be explained by younger or older leaves being better able to cope with heat stress and warrants further investigation.

For clonally reproducing seagrass meadows, epigenetic inheritance functions differently compared to plants with other life strategies due to the high level of mitotic reproduction [59]. In other clonally reproducing plants such as wild strawberry, environment associated methylation marks are inherited across clonal generations and can therefore contribute to adaptation [33]. In a study on *Arabidopsis*, the inheritance of organ-specific methylation mark following the generation of clonal offspring from somatic cells also indicates that epigenetics contribute to heritable variation in clonally reproducing plants [60]

During sexual reproduction, DNA methylation is more likely to be reset as methylation is erased during zygote formation [61], and in rice zygotes methylation is reprogrammed according to the levels of the mother (Q. [62,63]). However, some methylation marks can be inherited through plant sexual reproduction, for example variations deriving from spontaneous epimutations [64]. Epigenetic marks acquired during clonal propagation can also be maintained during sexual reproduction, as the epigenetic machinery cannot always reset such marks [65,60,66]. In this study, we identified differences in DNA methylation between younger and older clones of seagrass, but it is unknown whether these differences originated as accumulations of epimutations in the older plants, or occurred during clonal propagation. Hence, an experimental approach would be necessary to determine the heritability of these marks across clonal generations. However, our observations of differences in DNA methylation support the concept of epigenetics contributing to phenotypic variation in seagrass meadows with low genetic diversity, and their relevance to adaptation in clonal plants [28,33].

As all significantly enriched GO terms for the comparison of age groups occurred in TEs (Fig. 8), it is likely that the differential methylation between age groups mainly affects silencing of TEs and regulating TE mobility, which generally is an important role of DNA methylation (B. [67,68]). The functions of the affected TEs are not certain, but their assigned GO terms reveal the functions of closely located genes whose expressions may be affected: functions related to chromosome organization (GO:0051276, GO:0000793), double strand break repair (GO:0000742, GO:006302), and transcription regulation (GO:0045893, GO:0000981) (Fig. 8). Although our data cannot verify the connection, the enrichment of chromosome organization and transcription regulation related terms are consistent with the forming of a stress related memory in the epigenome [69].

A fast-ticking epigenetic clock is a possible explanation for epigenetic differences between vegetatively propagated seagrass [35]. Methylome differences between clones caused by the accumulation of stochastic methylation and demethylations, called spontaneous epimutations, can be associated with the divergence of plant clonal lines ([70]; van der Graaf et al., 2015; [34]). Our findings in this study did not align with the hypothesis of a fast-ticking epigenetic clock, because the epigenetic patterns were more influenced by age and tissue type rather than differentiation between the three ramet sections 1, 2 and 3. However, we cannot discount the possibility that the MethylRAD sequencing method only detects a limited number of sites [36], and may therefore not be sufficiently sensitive to any accumulation of random epimutations. Nevertheless, the larger epigenetic distance between the older samples

than younger samples (Fig. 10), although not statistically significant, is consistent with a gradual accumulation of epimutations. More detailed sequencing that allow to characterize DNA methylation fully at a finer scale across the genome is necessary to further examine the presence of an epigenetic clock.

This study advances our understanding of methylation variation among clonal seagrass shoots and between organs. Building upon this study's contribution, future work can relate DNA methylation marks with phenotypic traits to assess their functional effects in seagrass beyond the demonstrated link between DNA methylation and heat stress response [28]. Establishing these correlations paves the way for investigating causality using reverse epigenetic techniques, such as CRISPR based methods, to modify methylation marks and confirm their effect on the phenotype [71,72]. Identifying epigenetic control of resilience associated traits is particularly relevant for restoration of seagrass meadows with low genetic diversity dominated by few clones.

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CRediT authorship contribution statement

Anne M.L. Nilsen: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Galice Hoarau:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Irina Smolina:** Writing – review & editing, Methodology, Investigation. **James A. Coyer:** Writing – review & editing, Investigation. **Christoffer Boström:** Writing – review & editing, Methodology. **Martina E.L. Kopp:** Writing – review & editing, Investigation. **Alexander Jueterbock:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

DNA raw reads of this study are available at the European Nucleotide Archive Project PRJEB83852.

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Appendix A. Supplementary data

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