Epigenome-wide response to oil pollution in the foundation plant *Spartina alterniflora*

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Author Contributions

CLR & KJFV conceived the study. CLR, KJFV, MA, MR, MLA and AS designed the experiments and analyses. MA, MR, CAMW and TVG did the laboratory work. MA, MR, and TVG analyzed the epiGBS data. CLR, MA, DG, and AS analyzed the transcriptome and gene expression data. CLR, MA and MR wrote the first draft of the manuscript. All co-authors provided input and revisions to the manuscript.

Data accessibility

Raw data files are available on Dryad at XXX. Processed data and R scripts are available on github.com/alvarezmf/DWH_epigbs.

Abstract

Theory predicts that environmental challenges can shape the composition of populations, which is manifest at the molecular level. Previously, we demonstrated that oil pollution affected gene expression patterns and altered genetic variation in natural populations of the foundation salt marsh grass, *Spartina alterniflora*. Here, we used a reduced representation bisulfite sequencing approach, epigenotyping by sequencing (epiGBS), to examine relationships among DNA sequence, DNA methylation, gene expression, and exposure to oil pollution. We document genome-wide genetic and methylation differentiation between oil-exposed and unexposed populations, and a correlation of genome-wide methylation patterns and gene expression, independent of population genetic structure. In addition, we demonstrate genotypic differences in response to oil under controlled conditions. Overall, these findings demonstrate genetic variation correlated to oil pollution, and that variation in DNA methylation is abundant, responsive, and correlated to gene expression in natural populations, which may represent an important component of the response to environmental stress.

Introduction

Organismal interactions and response to environment are governed by molecular mechanisms, which are among the most basic levels of biological organization. Studies across a diversity of organisms have described the association of genetic variation with environmental factors (Feder & Mitchell-Olds, 2003; Andrew *et al.*, 2013). More recently, transcriptomics studies in natural populations have identified gene expression differences that underlie phenotypic plasticity, genotype-by-environment interactions, and local adaptation, and that some of these differences are only elicited in natural environments (Nicotra *et al.*, 2010; Nagano *et al*. 2012, 2019; Alvarez *et al.*, 2015). Hence, genetic variation and gene expression variation can translate into trait variation that contributes to organismal performance with important population- and community-level ecological effects (Whitham *et al.*, 2006; Hughes *et al.*, 2008; Schoener, 2011; Alvarez *et al*., 2015).

Additional layers of regulatory variation, including chromatin modifications, small RNAs, and other non-coding variants, can mediate changes in genotypic expression and phenotype. However, regulatory variation is infrequently studied in ecological settings (Nagano *et al*. 2012, 2019; Alvarez *et al*. 2015; Kudoh 2016; Richards *et al.*, 2017). Chromatin modifications such as DNA methylation can also vary among individuals within populations, and contribute to phenotypic variation through a variety of regulatory roles (Becker & Weigel, 2012; Jackson, 2017; Richards *et al.*, 2017). For example, DNA methylation may affect phenotype and subsequent ecological interactions by modulating the expression of genes (Alvarez *et al*., 2015, 2018), the types of transcripts that genes produce (Maor *et al.*, 2015), the movement of mobile elements (Matzke & Mosher, 2014), and the production of structural variants (Yelina *et al.*, 2015; Underwood *et al.*, 2018). At the same time, changes in genetic sequence or gene expression may cause variation in patterns of DNA methylation, creating a relationship that is bidirectional and heterogeneous across the genome (Secco *et al.*, 2015; Meng *et al.*, 2016; Niederhuth & Schmitz, 2017). Patterns of DNA methylation have been correlated to habitat types, exposure to stress, and shifts in species range (Verhoeven *et al*., 2010; Richards *et al*., 2012; Liebl *et al*., 2013; Xie & Li *et al*., 2015; Foust *et al*., 2016).

In 2010, the *Deepwater Horizon* (*DWH*) oil spill developed into the largest marine oil spill in history (National Commission on the BP *Deepwater Horizon* oil spill, 2011), and became an opportunity to test ecological and evolutionary hypotheses in a diversity of organisms exposed to this recurrent anthropogenic stress (e.g. Hazen *et al*. 2010; Whitehead *et al*. 2012;

108 Kimes *et al*. 2013, 2014; Robertson *et al.* 2017; Alvarez *et al.* 2018; DeLeo *et al.* 2018). A
109 mixture of crude oil and dispersants made landfall along 1,773 kilometers on the shorelines of
110 Louisiana, Mississippi, Alabama and Florida (Mendelssohn *et al.*, 2012; Michel *et al.,* 2013).
111 Nearly half of the affected habitat was salt marsh, which provides valuable ecosystem functions
112 such as providing nurseries for birds and fish, and buffering storm and wave action (Day *et al.*,
113 2007; Mendelssohn *et al.*, 2012; Michel *et al.,* 2013). Gulf of Mexico salt marshes are dominated
114 by the hexaploid foundation plant species *Spartina alterniflora* (2n=6x=62; Marchant, 1968),
115 which is remarkably resilient to a variety of environmental stressors (Pennings & Bertness, 2001;
116 Baisakh *et al.*, 2008; Baisakh & Subudhi, 2009; Silliman *et al.*, 2012; Bedre *et al.*, 2016; Armand
117 Cavé-Radet *et al.*, 2018). Crude oil exposure from the *DWH* oil spill resulted in reduced carbon
118 fixation, reduced transpiration, and extensive above-ground dieback in *S. alterniflora*
119 populations (Lin & Mendelssohn, 2012; Silliman *et al.,* 2012). While oil-affected populations
120 showed partial to complete recovery within seven months of the spill (Lin *et al.,* 2016), the
121 genomic and population level mechanisms that underlie this remarkable recovery have been
122 poorly characterized.
123 In previous studies, we found that pollution tolerance was correlated to changes in
124 expression of a diverse set of genes, including epigenetic regulators and chromatin modification
125 genes, such as a homolog of SUVH5 (Alvarez *et al.*, 2018). Despite the resilience of *S.*
126 *alterniflora* populations to the *DWH* spill, we also found evidence of genetic differentiation
127 between individuals from oil-exposed areas and nearby uncontaminated areas (Robertson *et al.*,
128 2017). We expected that DNA methylation patterns would be divergent between oil exposed and
129 unexposed populations, which might be induced by the environment or result from the genetic
130 differences between exposed and unexposed populations. However, while a few DNA
131 methylation loci (measured via methylation sensitive amplified fragment length polymorphism;
132 MS-AFLP) were correlated with oil exposure, we did not find genome-wide patterns in DNA
133 methylation correlated with oil exposure in *S. alterniflora* (Robertson *et al.*, 2017).
134 In this study, we used a recently developed reduced representation bisulfite sequencing
135 (RRBS) technique, epigenotyping by sequencing (epiGBS), to generate a more robust DNA
136 sequence and DNA methylation data set (van Gurp *et al.*, 2016). We expected that the increased
137 resolution, both in number and in detail of the markers, provided by this sequencing approach
138 would confirm our previously observed patterns of genetic differentiation, and allow us to
139 identify fine scale DNA methylation structure that was not apparent in our previous study. We

also predicted that we would find evidence that DNA methylation was correlated with changes in gene function since some fragments might overlap with the coding regions of genes (Niederhuth & Bewick *et al.*, 2016). In addition, we examined the potential for response to selection among genotypes to crude oil treatments in a greenhouse experiment. We expected that variation in response to crude oil among genotypes would indicate existing standing variation in wild populations of *S. alterniflora* that could be acted upon by selection.

Materials and Methods

Sample Collection

We collected individuals from the leading edge of the marsh at three contaminated and three uncontaminated sites near Grand Isle, Louisiana and Bay St. Louis, Mississippi in August 2010, four months after the *DWH* oil spill as described in previous studies (Table 1; Robertson *et al.,* 2017; Alvarez *et al.,* 2018). These sites were naturally variable in conditions, but all sites supported monocultures of *S. alterniflora*. Contaminated sites were identified by the visual presence of oil on the sediment, and substantial above-ground dieback of *S. alterniflora* on the leading edge of the marsh with *S. alterniflora* plants growing up through the dead wrack. Nearby uncontaminated sites did not have any visual signs of the presence of oil or noticeable dieback of the above ground portions of *S. alterniflora* (contamination status confirmed via NRDA databases, Robertson *et al.*, 2017). To standardize age and minimize developmental bias in sampling, we collected the third fully expanded leaf from each of eight individuals, spaced 10 meters apart at each of the six sites (N=48). Leaf samples were immediately frozen in liquid nitrogen to prevent degradation, and kept frozen during transport to the University of South Florida for processing and analysis.

Greenhouse oil response experiment

For the greenhouse experiment, we used 10 *S. alterniflora* individuals that had been collected from two oil-naïve sites ("Cabretta" and "Lighthouse") in the Sapelo Island National Estuarine Research Reserve in Georgia, USA, in May 2010. Collected plants were spaced ten meters apart, maximizing the chance that individuals were of different genotypes (Richards et al. 2004; Foust et al 2016). We grew these individuals in pots under greenhouse conditions for at least three years before beginning our experiments and propagated multiple replicates of each

172 genotype by rhizome. Individual ramets were separated and potted in 4 inch pots in a 50-50
173 mixture of peat and sand (Cypress Creek, Tampa, USA; Alvarez 2016).
174 We distributed six potted replicates of each of the 10 genotypes in each of two plastic
175 containment chambers, for a total of 60 biological samples. One chamber received only
176 uncontaminated fresh water, while the oil treatment chamber received 2.5% oil (sweet Louisiana
177 crude) in 62 liters of water, which we previously determined would induce strong phenotypic
178 response (Alvarez 2016). Tides were simulated once per day by filling containment chambers
179 with the water or water-oil mixture and allowing the fluid to drain into a catchment. We
180 measured biomass by tallying the number of living leaves and the number of living ramets when
181 the experiment began, and again seven days after crude oil was added.
182
183 *DNA extractions and library prep*
184 We isolated DNA from each sample (48) using the Qiagen DNeasy plant mini kit
185 according to the manufacturer's protocol. We prepared epiGBS libraries *sensu* van Gurp *et al.*
186 (2016). Briefly, isolated DNA was digested with the enzyme PstI, which is sensitive to CHG
187 methylation and biases resulting libraries toward coding regions (van Gurp *et al.,* 2016). After
188 digestion, adapters containing methylated cytosines and variable barcodes were ligated to either
189 end of the resulting fragments. We used the Zymo EZ Lightning methylation kit to bisulfite treat
190 and clean the DNA. Libraries were then amplified with the KAPA Uracil Hotstart Ready Mix
191 with the following PCR conditions: an initial denaturation step at 98ºC for 1 min followed by 16
192 cycles of 98ºC for 15s, 60ºC for 30s, and 72ºC for 30s, with a final extension of 72ºC for 5 min.
193 Paired-end reads were sequenced at the University of Florida Interdisciplinary Center for
194 Biotechnology Research on the Illumina HiSeq 5000.
195
196 *Data pre-processing and mapping to transcriptome*
197 We used the epiGBS pipeline (van Gurp *et al.,* 2016) to demultiplex samples, trim
198 adapter sequences, and assemble the *de novo* reference sequence
199 (https://github.com/thomasvangurp/epiGBS). To improve variant calling while accounting for
200 polyploidy, we realigned individuals to the *de novo* reference (generated from the epiGBS
201 pipeline), separately using BWAmeth (Pedersen *et al.,* 2014), and called variants using the
202 GATK pipeline (DePristo *et al.,* 2011) with a specified ploidy of six. Methylation was tabulated
203 using the existing epiGBS analysis pipeline. Both SNP and methylation data were filtered

separately to include only loci that were present in more than 90% of individuals, with no more than 50% missing from any one individual. We removed loci with less than 5x depth of coverage and remaining missing data were imputed via a k-nearest neighbors approach (impute, Hastie *et al.*, 2018).

All fragments were mapped to the published *S. alterniflora* transcriptome (Boutte *et al.,* 2016) and the *Oryza sativa* genome (Michigan State University version 7, Kawahara *et al.*, 2013) using BLAST (Altschul *et al.,* 1997). We estimated the number and order of exons by mapping the *S. alterniflora* transcriptome to *Oryza sativa*. We used BLAST (Altschul *et al.,* 1997) and RepeatExplorer (Novak *et al.,* 2013) to compare our sequenced fragments to the *S. alterniflora* transcriptome (Ferreira de Carvalho *et al.,* 2013, 2017; Boutte *et al.*, 2016) and known repeat elements, respectively.

Population genetics

This RRBS technique did not provide sufficient sequencing depth to estimate hexaploid genotype likelihoods with confidence, particularly considering the lack of a high-quality reference genome (Dufresne *et al.,* 2014; Boutte *et al.,* 2016). We therefore used the frequency of the most common allele within an individual at each polymorphic locus as a proxy to estimate Nei's genetic distance (StAMPP, Pembleton *et al.,* 2013).

We obtained pairwise F_{ST} values between populations to test for significant differentiation. We used distance-based redundancy analysis (RDA; Vegan, Oksanen *et al.* 2017) to minimize false positives (Meirmans 2015) in assessing isolation by distance using the formula (genetic distance ~ latitude * longitude).

To quantify the relationship between genome-wide variation and environmental conditions, we again used RDA with the formula (genetic distance ~ oil exposure + surrogate variables). RDA allowed us to assess the joint influence of all SNPs simultaneously (Meirmans 2015). We attempted to control for variation among sites with a replicated sampling strategy, but rather than using a single term for "population", we included parameters identified by surrogate variable analysis (SVA), which provides a method to account for residual variation due to unmeasured differences among populations, including environmental variation, life history variation, and geographical separation (Leek *et al.*, 2017). We estimated surrogate variables by fitting a full and null model to the allele frequency or DNA methylation frequency data matrix and examining the principal components of the residuals (Leek & Storey (2007). We used a

236 permutational test (999 permutations) to assess significance, and tested for differences in the
237 amount of dispersion around centroids among oil-exposed or unexposed populations
238 (PERMDISP2, Vegan, Oksanen *et al.,* 2017). We visualized results using multidimensional
239 scaling (MDS, Vegan, Oksanen *et al.,* 2017).
240 We used binomial linear mixed modeling (implemented in MACAU; Lea *et al.,* 2015)
241 directly on observed allele frequencies to identify individual SNPs that were significantly
242 correlated with oil contamination. For this analysis, we accounted for population structure with a
243 genetic relatedness matrix calculated as Nei's distance (StAMPP, Pembleton *et al*., 2013), and
244 used the surrogate variables as covariates. All p-values were corrected for family-wise false
245 discovery using the Q-value package in R (Storey *et al.,* 2015).
246
247 *Methylation analysis*
248 During the filtering process, loci were annotated with their methylation context, but all
249 contexts were pooled for distance-based analyses as well as family-wise error rate corrections
250 after locus-by-locus modeling. We tabulated methylation frequency at each locus (methylated
251 cytosines/(methylated+unmethylated cytosines)), and used methylation frequencies to generate a
252 Euclidean methylation distance matrix in R.
253 To identify signatures of DNA methylation variation that were correlated with oil
254 exposure while controlling for genetic structure, we used principal components analysis to
255 reduce the genetic data to orthogonal components, and identified 8 of the 42 possible principal
256 components axes that were significantly correlated with methylation. We then modeled the
257 impact of oil exposure to genome-wide patterns of DNA methylation by jointly assessing the
258 influence of all polymorphic methylation loci concurrently via RDA (Vegan, Oksanen *et al.*
259 2017) with the formula (methylation distance ~ oil exposure + surrogate variables + significant
260 principal components of allele frequency data). We corrected the resulting P values for false
261 discovery using a Holm correction, and visualized results using multidimensional scaling (MDS,
262 Vegan, Oksanen *et al.* 2017).
263 To identify differentially methylated positions (DMPs) between contaminated and
264 uncontaminated samples, we used binomial linear mixed modeling (MACAU; Lea *et al.,* 2015),
265 using the genetic relatedness matrix and surrogate variables as covariates to control for
266 population structure. We corrected locus-specific P-values for multiple testing (qvalue, Storey *et*
267 *al.,* 2015), and tested for overrepresentation of cytosine contexts (CG, CHG, CHH) using an

268 exact binomial test, implemented in R. Our epiGBS fragments rarely exceeded 200bp, and we
269 were unable to identify differentially methylated regions.
270
271 *Relationships to gene expression variation*
272 In a previous study using pools of individuals on a custom microarray, we found
273 differential expression in response to the *DWH* in 3,334 out of 15,950 genes that were assessed
274 in *S. alterniflora* (Alvarez *et al*., 2018). In order to make the epiGBS data comparable to our
275 pooled microarray design, we concatenated SNPs and methylation polymorphisms from
276 individuals into *in silico* sample pools by averaging values at individual loci across the same
277 three individuals within pools that were used in the gene expression analysis. We then calculated
278 genetic, expression, and methylation distances between sample pools and used multiple
279 regression on distance matrices to assess the relationship between all three data types (MRM;
280 ecodist, Goslee & Urban, 2007).
281 We assessed the correlation between gene expression, genetic variation, and methylation
282 variation within the same fragment (i.e. in *cis*) gene-by-gene using only microarray probes and
283 epiGBS fragments that could be mapped to the *S. alterniflora* transcriptome. We regressed the
284 expression values of each gene against the methylation frequencies and most common allele
285 frequencies at corresponding epiGBS fragments using the *lm* function in R with the formula
286 (gene expression ~ *cis* methylation + *cis* SNP). We summarized variation across each epiGBS
287 fragment for multiple SNP or methylation loci by using the first principal component of each
288 data type. We interpreted significant effects of SNPs or DNA methylation to mean that the
289 expression of a given gene depended on the frequency of the nucleotide or methylation
290 polymorphism. We corrected for false discovery using qvalue (Storey *et al.*, 2015).
291
292 *Greenhouse statistical analysis*
293 We used linear mixed models (lme4; Bates *et al*. 2015) to analyze the change in above-
294 ground biomass over the course of the experiment (measured as the change in number of leaves
295 overall and in the number of leaves per ramet), as biomass is a reliable proxy of fitness across
296 plants (Younginger *et al*. 2017). Each model was written as (Phenotype ~ Treatment * Genotype,
297 where asterisks represent main effects and all interactions. We do not explicitly test for
298 differences between sites since admixture is high between sites on Sapelo Island and we found
299 no evidence of genetic differentiation (Foust *et al*. 2016). We assessed significance for main

300 effects and interactions using type III tests. However, to identify individual genotypes
301 responding more strongly than others, we conducted post-hoc pairwise comparisons using a
302 Tukey's correction (emmeans; Lenth 2018), which provide additional power to test responses in
303 individual genotypes (Hsu 1996).
304
305 **Results**
306
307 *epiGBS yields informative genetic and methylation loci*
308 Following library preparation, we removed four samples due to stochastic under-
309 sequencing. The libraries for 44 individuals (Table 1) generated 6,809,826 total raw sequencing
310 reads, of which 3,833,653 (56.3%) could be matched to their original mate strand. *De novo*
311 assembly using the epiGBS pipeline resulted in 36,131 contiguous fragments (19-202 basepairs)
312 with a total length of 5,441,437 bp (Figs. **S1, S2**). Given the size of the *S. alterniflora* genome
313 (2C values = 4.3 pg; Fortune *et al.*, 2008), our epiGBS approach assayed approximately 0.3% of
314 the genome. However, fragments that were >90% similar were merged, and polyploid
315 homeologs may have been concatenated. With BLAST, we found 10,103 fragments mapped to
316 2,718 transcripts in *S. alterniflora* transcriptome. We found that 1,571 transcripts (57.8%)
317 contained multiple epiGBS fragments that align to the same place, and 296 (10.9%) contained
318 multiple epiGBS fragments that mapped to different places within the same gene. We suspect
319 that multiple epiGBS fragments map to the same location because some epiGBS contiguous
320 fragments represent different homeologs of the same region, which mapped to the same location.
321 Only 1% of reads map to repetitive elements, confirming that *Pst1*-fragmented libraries were
322 biased away from highly methylated, repetitive regions (van Gurp *et al.,* 2016).
323
324 *Genetic differentiation*
325 Our initial sequencing run yielded 171,205 SNPs across all individuals. After filtering to
326 common loci and removing invariant sites, we imputed 10-25% missing genetic data and 10-50%
327 methylation data per sample (Fig. **S3**), resulting in 6,521 SNP loci. Significant data loss after
328 filtering was likely due to insufficient sampling depth across an exceptionally large hexaploid
329 genome. Of these 6,521 loci, 243 SNPs occurred in transcripts. Pairwise F_{ST} calculations showed
330 that all sites were significantly different from each other (Table 3), with no evidence of isolation
331 by distance (P>0.05 for latitude, longitude, and interaction). As in our AFLP study, we found

332 significant genetic differentiation that was correlated to oil exposure: oil exposure explained
333 7.9% of the variance in DNA sequence (P=0.035, Table 2). These results are recapitulated in the
334 MDS visualization, which shows partial separation of oil-exposed and unexposed sites along
335 MDS axis 1 (Fig. **1**). We found 42 SNPs that were significantly associated with oil exposure,
336 including 6 that overlapped with the *S. alterniflora* transcriptome. Four of these genes were
337 annotated in *O. sativa* (Table S1). After testing for homogeneity of group dispersion, we found
338 no evidence of change in variance in oil-exposed populations (P=0.696).
339
340 *DNA methylation differentiation*
341 Our bisulfite sequencing yielded 1,402,083 cytosines that were polymorphic for
342 methylation across our samples before filtering. After filtering our data to common loci as
343 described above, we analyzed 19,577 polymorphic methylated loci, 5,836 of which occurred in
344 the CG context, 5,069 in the CHG context, and 8,673 in the CHH context. These proportions of
345 polymorphic loci did not change significantly due to oil exposure (Fig. **2**). Methylation calls
346 were collapsed for symmetric CG and CHG loci across "watson" and "crick" strands so that
347 methylation on either one or both strands was considered as a single locus. A quarter of cytosines
348 that showed methylation polymorphism (26.3%) were methylated in fewer than 5 individuals,
349 suggesting that many cytosines were infrequently methylated or methylated only on some sub-
350 genomes. We found significant methylation differentiation by oil exposure after controlling for
351 population structure with principal components of genetic data (P=0.022). Oil explained 9.2% of
352 the variation in methylation.
353 We found 14 differentially methylated positions (DMPs between exposure types
354 (Q<0.05, Fig. **3**; Table S1). These loci occurred nearly equally in CG (4 loci), CHG (5 loci), and
355 CHH (5 loci) contexts, and no contexts were overrepresented relative to their distribution across
356 all loci (P>0.2 for all tests). Among these significant loci, one was located within a fragment that
357 mapped to the *S. alterniflora* transcriptome. In addition, four were located in one of three
358 fragments that mapped to the *Oryza sativa* transcriptome (Table S1). Most of these 14 loci had
359 negligible differences in the magnitude of methylation frequency changes (average 2.8% change
360 between exposed and unexposed populations). However, we found a 30% change in methylation
361 frequency of a locus located in an actin filament depolymerization protein (Os03g56790; ADF2).
362 We found no DMPs that mapped to the first 250 nucleotides of exon 1 of any gene, which is

363 where DNA methylation may be particularly relevant to differential expression (Secco *et al.*,
364 2015).
365
366 *Correlations with gene expression*
367 Although 2,718 epiGBS fragments mapped to the *Spartina* transcriptome, none of these
368 fragments overlapped with transcripts that showed expression differences correlated with
369 exposure to the *DWH* oil spill (Alvarez *et al.*, 2018). We found no significant relationship
370 between genetic distance and gene expression distance (P=0.185, Mantel's R=0.1489), but a
371 moderate correlation between patterns of methylation variation and genome wide gene
372 expression (P=0.009, Mantel's R=0.2319). We had expression data and either SNPs or
373 methylation polymorphisms for only 54 genes, and we found no association between individual
374 SNPs or methylation polymorphisms with expression in *cis*.
375
376 *S. alterniflora genotypes differ in their response to crude oil*
377 We found a significant effect of oil exposure on both the change in leaves (F= 13.09, P <
378 0.001) and the change in leaves normalized by the number of ramets (F = 28.75, P < 0.001).
379 Type III tests did not show significant differences among genotypoes in oil response; however
380 post-hoc comparisons identified three genotypes (C, G, J; P<0.05, Table S2) that lost a
381 significantly greater number of leaves over the course of the experiment relative to other
382 genotypes, and one genotype (H; P<0.05) that lost significantly more leaves when normalized by
383 the number of ramets (Table S2).
384
385 **Discussion**
386 *Spartina alterniflora* displays high levels of genetic and methylation variation across
387 environmental conditions in its native range (Richards *et al.*, 2004; Hughes & Lotterhos, 2014;
388 Foust *et al.,* 2016; Robertson *et al.*, 2017), potentially providing substrate for both genetic and
389 epigenetic response to pollution. We previously found that genetic structure and expression of
390 3,334 genes were correlated to exposure to the *DWH* oil spill, but genome-wide methylation
391 variation was not (Robertson *et al.,* 2017; Alvarez *et al.*, 2018). Higher resolution epiGBS
392 suggests that both genetic sequence and DNA methylation are correlated with crude oil exposure
393 in *S. alterniflora*, and that changes in gene expression are more strongly correlated to genome-
394 wide patterns of DNA methylation than population structure. Additionally, our greenhouse

395 experiment shows genotypic variation in crude oil response, as measured by reduction in
396 biomass. These findings are consistent with genotype-specific mortality, and suggest that the
397 *DWH* oil spill may have been a selective event.
398
399 *Confirmation of genetic and epigenetic response to the DWH*
400 We found significant genetic differentiation between oil-exposed and unexposed sites,
401 which may reflect either stochastic mortality in oil-exposed areas from a severe bottleneck, or a
402 signature of selection for oil tolerance in affected populations. *Spartina alterniflora* is a highly
403 stress tolerant plant, and populations have persisted after exposure to the *DWH* oil spill, even
404 after extensive dieback (Lin & Mendelssohn, 2012; Silliman *et al.*, 2012; Lin *et al.*, 2016).
405 However, our genetic data and our greenhouse experiment suggest that some *S. alterniflora*
406 genotypes were more susceptible than others to crude oil stress, and either had not regrown at the
407 time of sampling or experienced mortality as a result of oil exposure. Our genetic marker studies,
408 genotypic differences in our greenhouse study and previous accounts of initial losses in live
409 aboveground and belowground biomass, support the hypothesis of differential mortality (Lin *et*
410 *al*., 2016). Although we found no evidence for a reduction in genetic variation, which may
411 further indicate selection for tolerant genotypes, the high ploidy of *S. alterniflora* makes accurate
412 quantification of total genetic variation challenging. Further investigations will be required to
413 confirm whether mortality varied by genotype in oil-exposed populations, the magnitude of
414 selection and if there was reduction in genetic variation among oil-exposed populations.
415 DNA methylation differences that were correlated to oil exposure in our study may
416 reflect either the downstream effects of genetic variants, an induced regulatory response to oil
417 exposure, or both (Meng et al. 2016). For example, in another study of *S. alterniflora*
418 populations, patterns of DNA methylation were more strongly correlated than genetic structure
419 with microhabitat, and some response of DNA methylation to environment was independent of
420 population structure (Foust *et al.,* 2016). In the oil contaminated populations, we identified a
421 differentially expressed gene that was homologous to the histone methyltransferase SUVH5,
422 which may modulate fitness effects during oil exposure (Alvarez *et al.,* 2018). Histone
423 methylation is linked to DNA methylation through the regulation of *DNA (cytosine-5)-*
424 *methyltransferase CMT3* activity (Stroud *et al.*, 2013). Therefore, we hypothesized that the
425 differential expression of SUVH5 in response to crude oil exposure would result in differences in
426 DNA methylation. With the increased resolution offered by epiGBS (19,615 methylation loci

427 compared to 39 polymorphic MS-AFLP fragments), we found a genome-wide signature of
428 methylation differentiation between oil affected and unaffected sites after controlling for
429 population structure. In our greenhouse experiment, all genotypes responded to oil exposure by
430 reducing biomass. However, some genotypes responded more dramatically, such that crude oil
431 exposure may select on standing variation, despite broad plastic response across genotypes.
432
433 *Epigenetic variation is correlated to genome wide patterns of gene expression*
434 We expected that our fragments would be biased toward coding regions of the genome,
435 and that we would find DNA methylation was correlated with changes in gene function where
436 fragments overlapped with the promoters or the 5' end of the coding regions of genes. We found
437 that almost 1/3 (10,103 of the 36,131) of the epiGBS fragments mapped to 2,718 transcripts in
438 the *S. alterniflora* transcriptome. However, none of these 2,718 genes overlapped with the 3,334
439 oil responsive genes from our previous transcriptome analysis (Alvarez *et al.*, 2018), and few
440 fragments overlapped the 5' end of genes. Additionally, our data was limited to explore an
441 association between DNA methylation and expression when individual fragments were
442 examined. Therefore, our expectation that we could correlate local methylation polymorphisms
443 to expression of individual oil responsive genes was not fulfilled in this study. However,
444 genome-wide patterns of DNA methylation are more strongly correlated than genetic structure
445 with gene expression, suggesting a broader interplay between the regulation of gene expression
446 and DNA methylation.
447 Combined, our results suggest that genetic variation and regulatory mechanisms may play
448 a role in the response of *S. alterniflora* populations to crude oil pollution. Although we are
449 unable to dissect the mechanistic role of these molecular processes, our data suggests that crude
450 oil exposure may have generated differentiation between oil exposed and unexposed populations,
451 which is manifest in patterns of genetic variation, gene expression and DNA methylation.
452 Variation in these genome-level processes may modulate both the fixed and plastic phenotype
453 responses to crude oil exposure in this system. Our greenhouse study supports the hypothesis that
454 both plasticity, in the form of oil tolerance, as well as differential mortality may shape the
455 evolutionary outcome of *S. alterniflora* populations exposed to crude oil. Reverse genetic screens
456 and higher resolution surveys may help to discern the mechanistic roles of methylation and
457 genetic variation in genes of interest in oil-exposed populations.
458

459 *Reduced representation sequencing compared to AFLP*

460 As the field of ecological genomics matures, there is a pressing need to develop robust
461 assays and statistically sound measures of population regulatory variation. Reduced
462 representation methylation sequencing techniques are attractive for ecological epigenomics
463 because they can be used to infer genome wide patterns of both genetic and methylation
464 variation without a high-quality reference genome (Robertson & Richards, 2015; Richards *et al.*,
465 2017; Paun *et al.*, 2019). However, they still have serious limitations particularly for species that
466 do not yet have a fully sequenced reference genome (Paun *et al.*, 2019).

467 We assessed the utility of epiGBS compared to MS-AFLP, and expected that the
468 substantial increase in markers would confirm our results from our MS-AFLP study and lend
469 greater resolution to detect patterns of DNA methylation variation. Our epiGBS survey detected
470 significant differentiation in both genetic variation and DNA methylation that was correlated to
471 oil exposure, suggesting that epiGBS provides increased resolution over MS-AFLP to detect
472 genome-wide methylation differences in studies of similar sample sizes. However, despite the
473 much larger data set generated by epiGBS compared to MS-AFLP, we only found 14
474 differentially methylated positions, which is not substantial improvement over the five loci
475 detected by MS-AFLP (Robertson *et al.*, 2017). The comparable number of DMPs and lack of
476 power to evaluate *cis* association between methylation, genetic variation, and gene expression
477 indicate that the ability to characterize individual functional elements remains obscured by
478 substantial variation in methylation, and is limited by the small fraction (an estimated 0.3%) of
479 the genome that is assayed. Additionally, we found few fragments that overlapped the
480 functionally relevant 5' end of transcripts. Without a reference genome for *S. alterniflora,* we
481 were unable to identify fragments that overlapped promoter regions.

482 Sequencing-based techniques provide the potential to identify functional genomic
483 regions, but correct annotations rely on genomic resources in a relevant reference. In polyploid
484 species like *S. alterniflora*, a greater number of duplicated copies and the potential for
485 neofunctionalization among them creates additional uncertainty for using annotations from other
486 species (Primmer *et al.*, 2013). *Spartina alterniflora* has various levels of duplicated gene
487 retention and homeologous expression (Ainouche *et al.*, 2003; Fortune *et al.*, 2007; Ferreira de
488 Carvalho, 2013, 2017; Boutte *et al.*, 2016), which may result in more opportunities for gene
489 diversification and subfunctionalization (Chen *et al.*, 2015; Salmon & Ainouche, 2015; Shimizu-
490 Inatsugi *et al.* 2017), and complicates gene annotation. Therefore, while studies with RRBS

491 techniques in non-model plants offer increased power to detect broad, genome-wide patterns of
492 variation that may be correlated to ecology, they are still limited for the detection of specific
493 gene function. Future studies will benefit from optimizing protocols that enrich for specific
494 portions of the genome (Heer et al. 2018), but generating a draft reference genome will be
495 imperative to allow for better exploitation of RRBS data and ascertain gene function (Paun *et al.*,
496 2019). Improving genomics resources in a variety of organisms is an essential next step for
497 understanding the molecular level basis of ecological interactions.
498

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507 **Tables and Figures**

508

509 **Table 1** Sampling information across all sites after filtering.

Site Location	Site Code	Exposure	No. of individuals
Grand Isle, LA	GIN1	None	7
Grand Isle, LA	GIN2	None	7
Barataria Bay, LA	GIO1	Heavily Oiled	8
Barataria Bay, LA	GIO2	Heavily Oiled	8
Bay St. Louis, MS	MSN	None	7
Bay St. Louis, MS	MSO	Heavily Oiled	7

510

511 Note: Site information includes location and whether oil was observed at each site (exposure).

512 **Table 2** Association between oil exposure, genetic distance, and methylation distance across
513 tests.

		Genetic		Methylation	
Factor	df	F	Variance explained	F	Variance explained
oil	1	1.395 *	0.079	1.960*	0.092

514

515 Notes: Test statistics and significance, determined through RDA. For clarity, we do not show
516 significance for estimated surrogate variables (7 for methylation data, 16 for genetic data) or the
517 8 principal components of allele frequencies for the methylation data. $P * \leq 0.05$

518 **Table 3** Pairwise Fst among three oil contaminated and three uncontaminated sites. Bold (i.e. all
519 entries) indicates significance at P<0.001.

	GIN1	GIN2	MSN	GIO1	GIO2
GIN2	**0.1304**				
MSN	**0.1329**	**0.1421**			
GIO1	**0.1451**	**0.1264**	**0.1382**		
GIO2	**0.0966**	**0.1020**	**0.1198**	**0.1158**	
MSO	**0.1171**	**0.1471**	**0.1497**	**0.1580**	**0.1008**

520

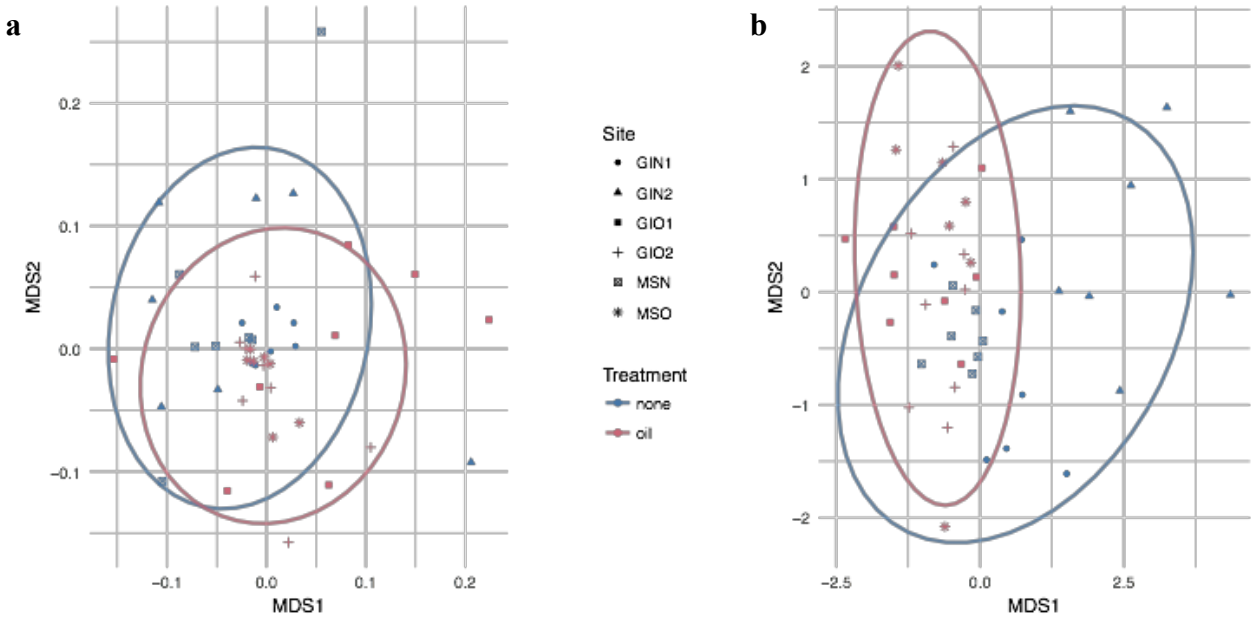


Figure 1 Multidimensional scaling (MDS) of **a**) genetic distance matrix and **b**) DNA methylation distance matrix. Circles represent 95% confidence intervals around the centroid of each group: no oil exposure ("none") or oil-exposed ("oil").

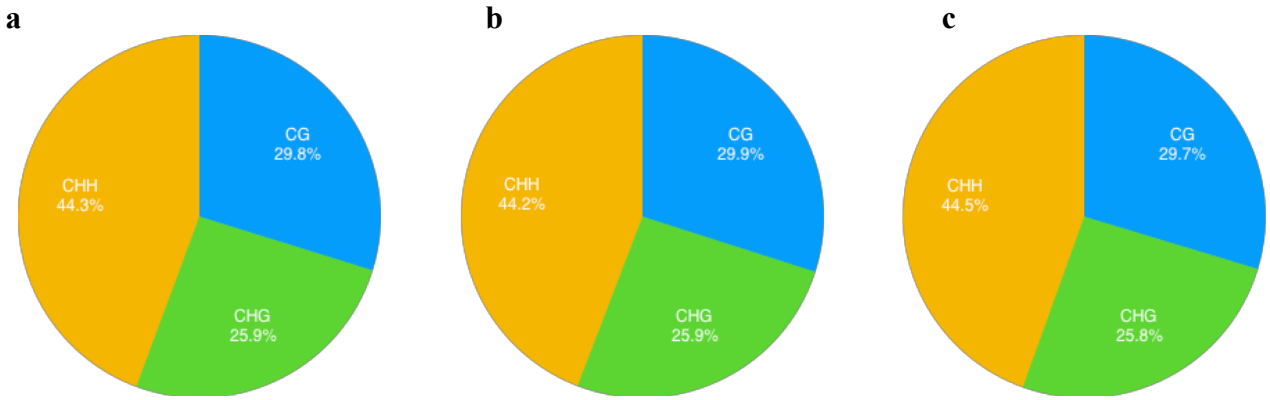


Figure 2 Proportion of methylated sites by context across a) all samples, b) in uncontaminated sites and c) oil-contaminated sites.

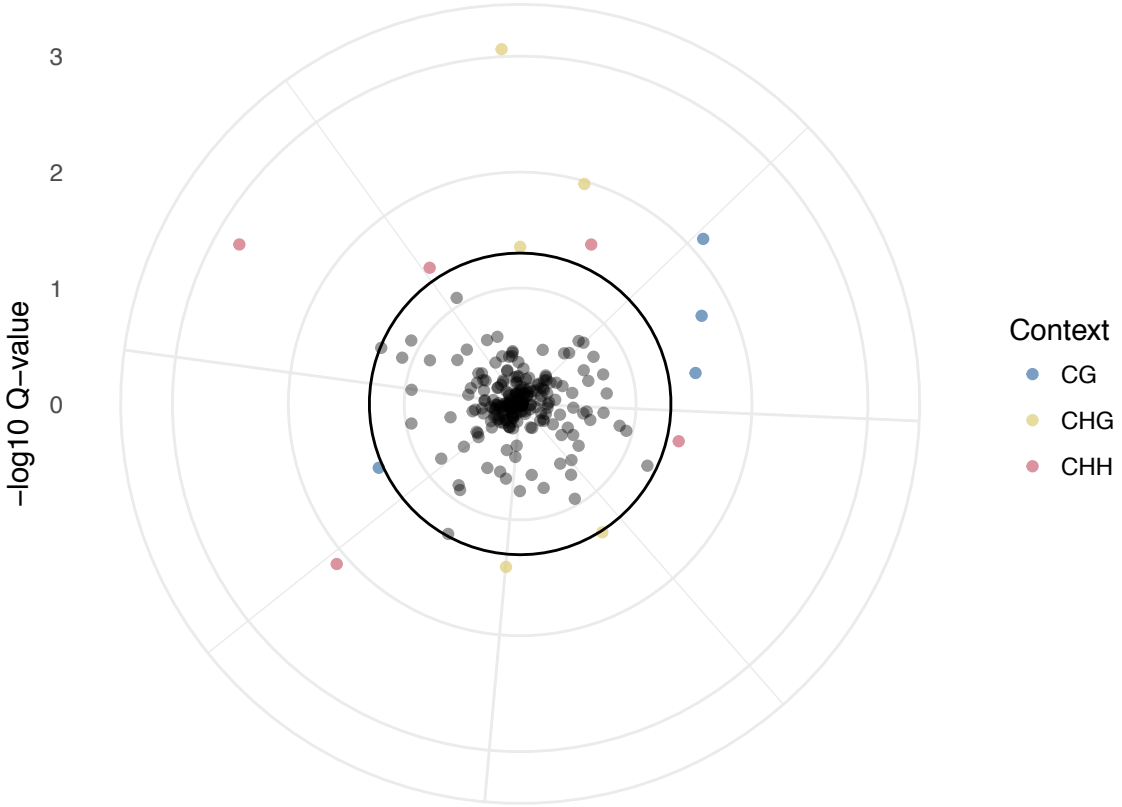


Figure 3 Circular manhattan plot showing 14 significant outlier methylation loci, as determined by Q-value. Only significant loci (Q<0.05) are colored, where colors represent trinucleotide context. Plot is shown as circular to accommodate unknown positions, as *S. alterniflora* does not have a reference genome.

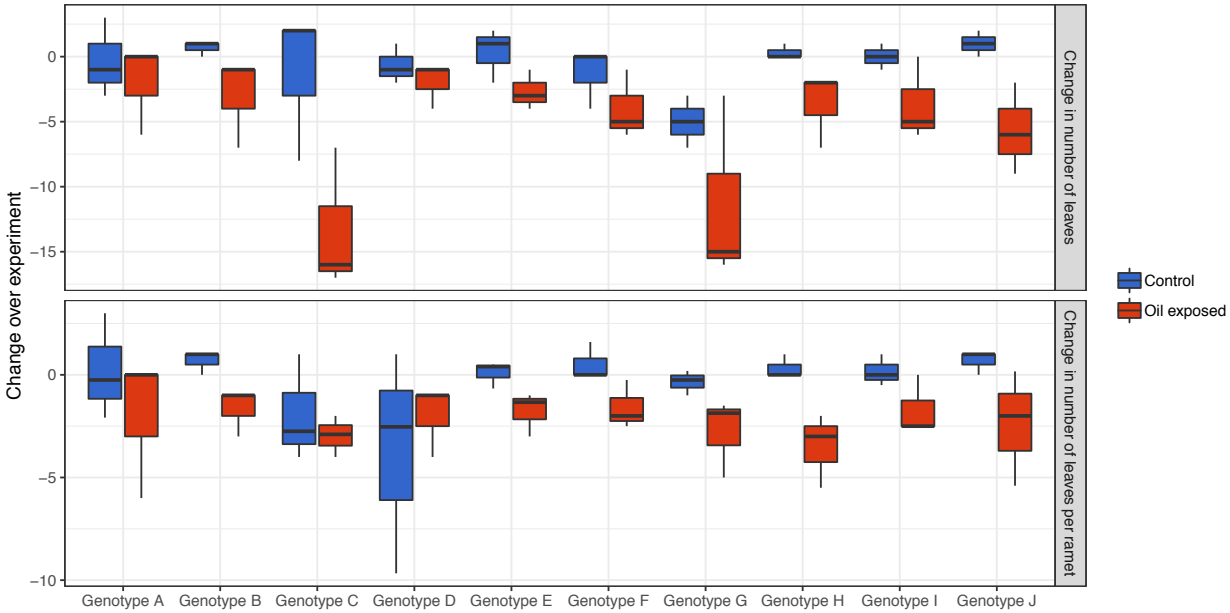


Figure 4 Results of greenhouse exposure. Note that scales differ by phenotype measured, and represent the change in count number over the course of the experiment.

542 **Table S1** Genetic and methylation loci significantly differentially associated with oil exposure.

Contiguous fragment	Position	Context	Q-value	*O. sativa* annotation	Gene name
3386	141	-	0.0056		
5449	67	-	0.0399		
7242	26	-	0.0447		
8158	95	-	0.016		
9488	45	-	0.0339		
9488	51	-	0.0421		
9488	94	-	0.0385		
10931	43	-	0.0385		
11792	111	-	0.0339		
11939	123	-	0.0033	Os06g02570	Syntaxin, putative
11972	49	-	0.0327		
11974	102	-	0.012		
11974	131	-	0.0327		
12084	26	-	0.0447		
12418	86	-	0.0033		
13976	47	-	0.0385		
14656	84	-	0.0385		
14804	93	-	0.0001		
14957	105	-	0.012		
19335	34	-	0.0032		
19759	76	-	0.0447		
26805	111	-	0.0466		
26906	41	-	0.0009		
27016	91	-	0.0421	Os03g18220	pyruvate decarboxylase isozyme 2, putative
27227	103	-	0.0307		
28167	59	-	0.0443		
28937	126	-	0.0421		
28937	133	-	0.0413		
28937	147	-	0.0307		
30433	105	-	0.013		
31126	85	-	0.0181		
31126	147	-	0.0174		
31604	78	-	0.0421		
32215	52	-	0.0033		
33738	102	-	0.0218		
34288	53	-	0.0001	Os11g39230	retrotransposon protein, putative
34398	88	-	0.0013		
34744	123	-	0.0366		

35646	61	-	0	Os01g43070	psbP-related thylakoid lumenal protein 4, chloroplast precursor, putative
36084	92	-	0.0009		
36084	96	-	0.0447		
36084	115	-	0.0308		
673	43	CG	0.0461		
8098	139	CG	0.0291		
11959	13	CG	0.0182	Os01g69980	TCP family transcription factor, putative
34711	123	CG	0.0075		
183	10	CHG	0.0443		
527	19	CHG	0.0388	Os03g56790	actin-depolymerizing factor, putative
4191	107	CHG	0.0105		
5540	171	CHG	0.0482		
7334	97	CHG	0.0009		
45	82	CHH	0.0388	Os03g51920	peptidase, M50 family, putative
5540	34	CHH	0.008		
6013	69	CHH	0.0016	Os10g34480	cytochrome P450, putative
14227	11	CHH	0.0312	Os08g38560	receptor-like protein kinase 2 precursor, putative
14776	15	CHH	0.0394		

543

544 Only loci with Q-value <0.05 are shown. Trinucleotide context (CG, CHG, CHH) shown for
545 methylated loci. Gene numbers from *O. sativa* are shown for loci on fragments that were able to
546 annotated.

547 **Table S2** Post-hoc tests of individual genotypes, based on estimated marginal means, in our
548 greenhouse experiment.

Phenotype	Genotype	df	T-ratio
Change in leaves per ramet	Genotype A	40	1.337
Change in leaves per ramet	Genotype B	40	1.403
Change in leaves per ramet	Genotype C	40	0.632
Change in leaves per ramet	Genotype D	40	-1.043
Change in leaves per ramet	Genotype E	40	1.116
Change in leaves per ramet	Genotype F	40	1.273
Change in leaves per ramet	Genotype G	40	1.465
Change in leaves per ramet	Genotype H	40	2.306*
Change in leaves per ramet	Genotype I	40	1.103
Change in leaves per ramet	Genotype J	40	1.851
Change in leaf number	Genotype A	40	0.628
Change in leaf number	Genotype B	40	1.381
Change in leaf number	Genotype C	40	4.521***
Change in leaf number	Genotype D	40	0.502
Change in leaf number	Genotype E	40	1.130
Change in leaf number	Genotype F	40	1.005
Change in leaf number	Genotype G	40	2.386*
Change in leaf number	Genotype H	40	1.507
Change in leaf number	Genotype I	40	1.381
Change in leaf number	Genotype J	40	2.512*

549

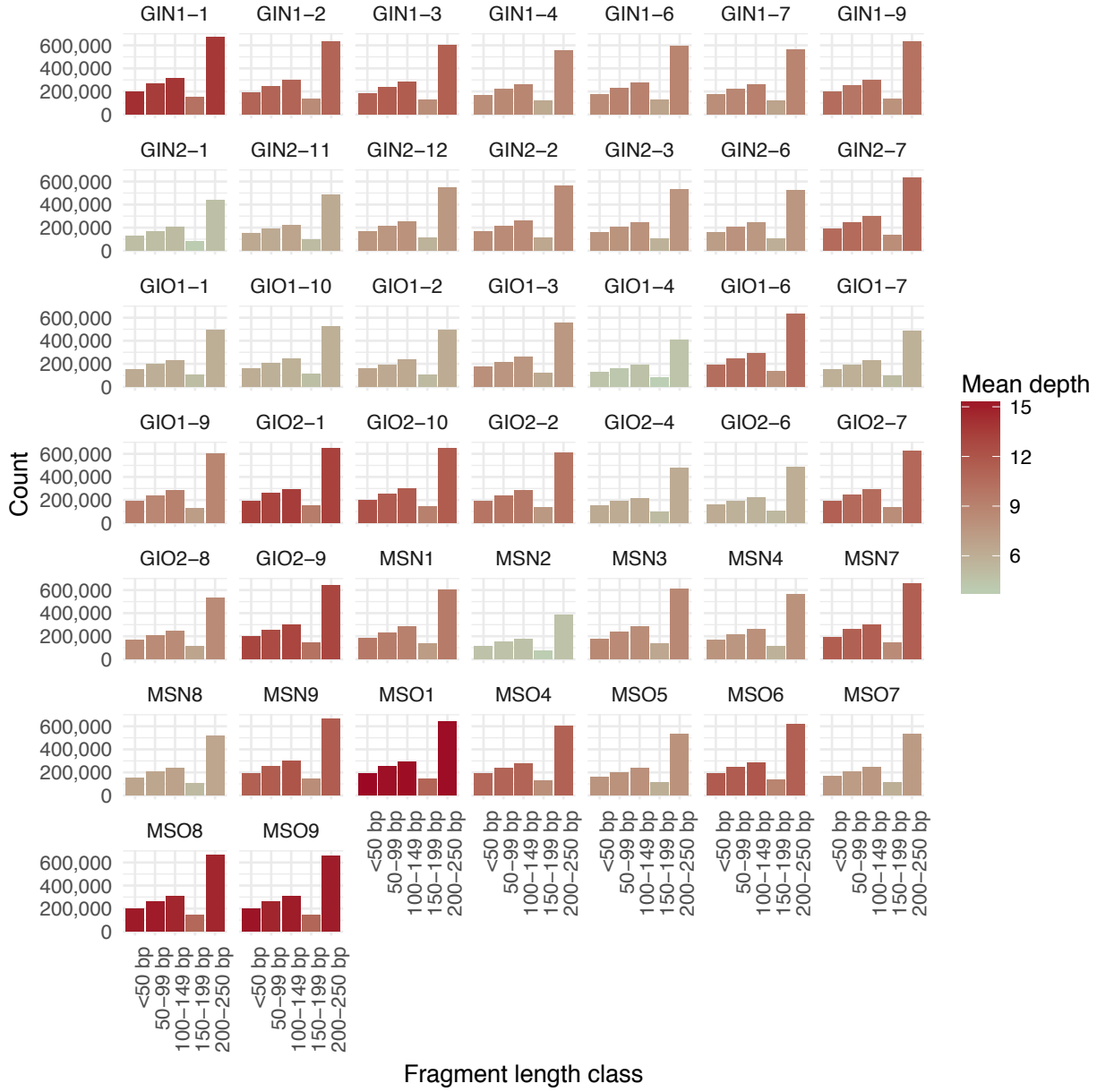


Figure S1 Depth of sampling by fragment size in each sample across all fragments. Fragments ranged from 19-202 basepairs (bp) for a total length of 5,441,437 bp (approximately 0.3% of the 2117 Mbp *S. alterniflora* genome (2C= 4.33pg); Fortune *et al.,* 2008). Fragments shown in classes on the X axis.

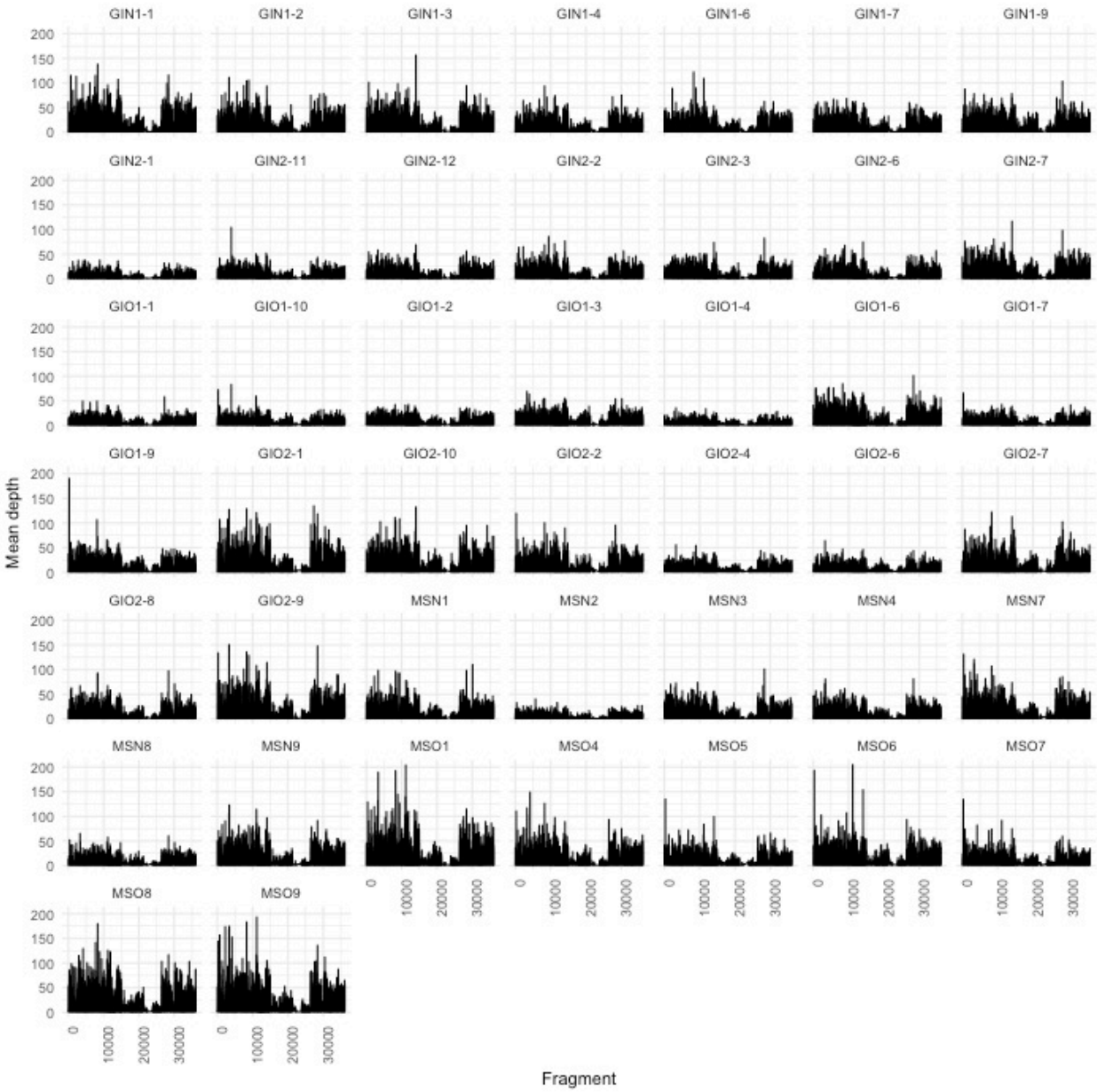
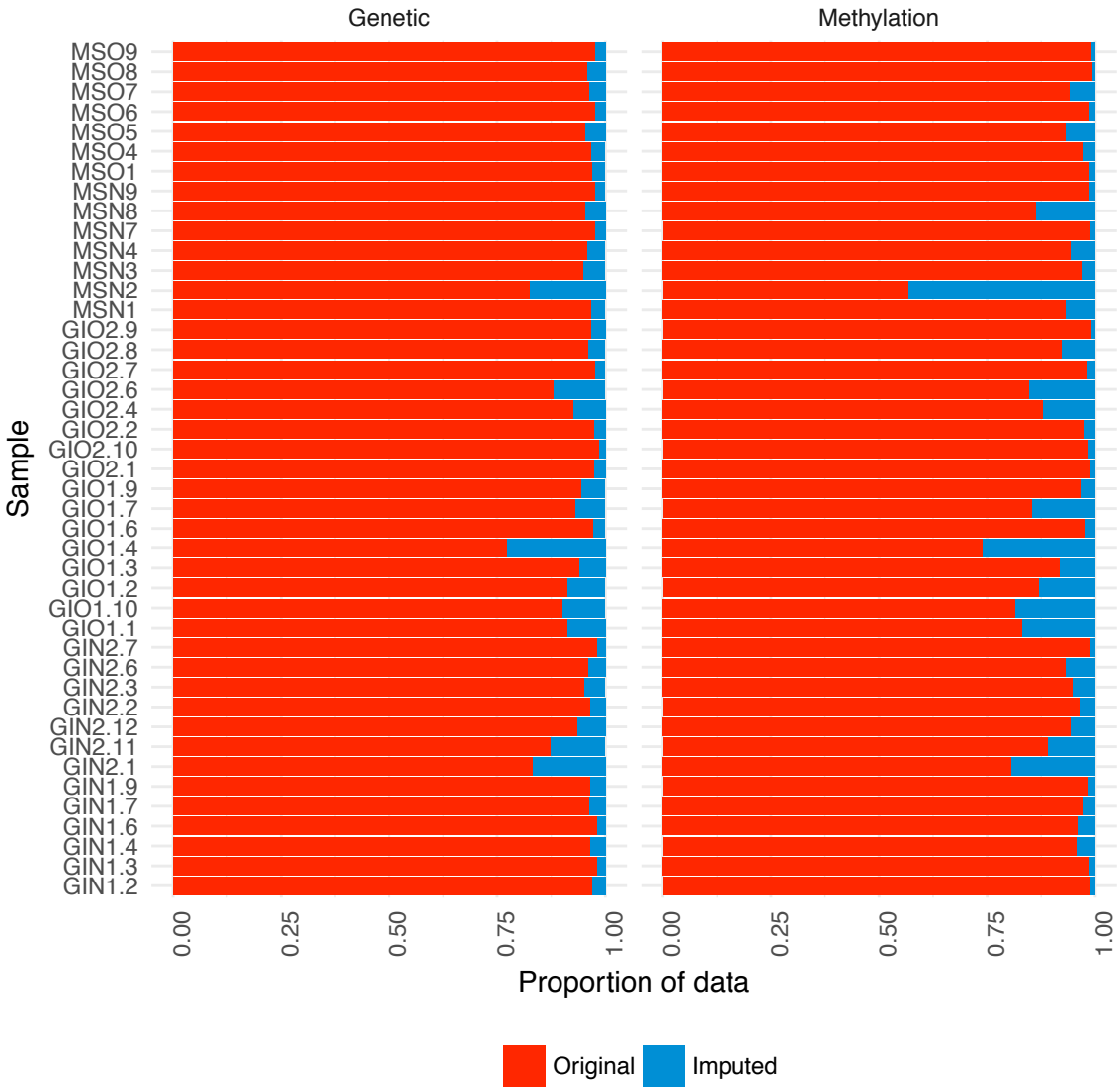


Figure S2 Sequencing depth for each sample for each of the 36,131 fragments.

561



562

563 **Figure S3.** Missing and imputed data by sample after filtering. Genetic and methylation data

564 were both filtered to include only loci present in >90% of samples and samples with less than

565 50% missing data.

566

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