Reduced-representation bisulfite sequencing finds 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

The application of genomics technology in ecological contexts allows for examination of how rapid environmental change may shape standing molecular level variation and organismal response. We previously demonstrated an effect of oil pollution on gene expression patterns and genetic variation, but not methylation variation, in oil-exposed 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. With the increased resolution of epiGBS, we document 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. Overall, these findings demonstrate that variation in DNA methylation is abundant, responsive, and correlated to gene expression in natural populations, and may represent an important component of the response to environmental stress.

75 **Introduction**

76 The application of molecular techniques to ecological questions can provide insight into
77 the mechanisms that govern ecological interactions at the most basic levels of biological
78 organization. Studies across a diversity of organisms have described the association of genetic
79 variation with environmental factors (Feder & Mitchell-Olds, 2003; Andrew *et al.*, 2013). More
80 recently, transcriptomics studies in natural populations have identified gene expression
81 differences that underlie phenotypic plasticity, genotype-by-environment interactions, and local
82 adaptation, and that some of these differences are only elicited in natural environments (Nicotra
83 *et al.*, 2010; Andrew *et al.*, 2013; Alvarez *et al.*, 2015). Hence, genetic variation and gene
84 expression variation can translate into trait variation that contributes to organismal performance
85 with important population- and community-level ecological effects (Whitham *et al.*, 2006;
86 Hughes *et al.*, 2008; Schoener, 2011; Alvarez *et al*., 2015). Additional layers of regulatory
87 variation, including chromatin modifications, small RNAs, and other non-coding variants, can
88 mediate these changes in genotypic expression and phenotype, but are infrequently studied in
89 ecological settings (Nicotra *et al.*, 2010; Richards *et al.*, 2017).

90 Like genetic variation and gene expression variation, chromatin modifications such as
91 DNA methylation can also vary among individuals within populations, and contribute to
92 phenotypic variation through a variety of regulatory roles (Becker & Weigel, 2012; Jackson,
93 2017; Richards *et al.*, 2017). For example, DNA methylation may affect phenotype and
94 subsequent ecological interactions by modulating the expression of genes (Alvarez *et al*., 2015,
95 2018), the types of transcripts that genes produce (Maor *et al.*, 2015), the movement of mobile
96 elements (Matzke & Mosher, 2014), and the production of structural variants (Yelina *et al.*,
97 2015; Underwood *et al.*, 2018). On the other hand, changes in genetic sequence or gene
98 expression may cause variation in patterns of DNA methylation, creating a relationship that is
99 bidirectional and heterogeneous across the genome (Secco *et al.*, 2015; Meng *et al.*, 2016;
100 Niederhuth & Schmitz, 2017). DNA methylation can vary in response to environment, and has
101 been correlated to habitat types, exposure to stress, and shifts in species range (Verhoeven *et al*.,
102 2010; Richards *et al*., 2012a; Liebl *et al*., 2013; Xie & Li *et al*., 2015; Foust *et al*., 2016). While
103 some studies have provided insight into how the complex interaction between genetic variation,
104 DNA methylation, and the regulation of gene expression translates into organismal response (e.g
105 Wibowo *et al.* 2016), these mechanisms are only understood in a few model species, partly

106 because of the limited genomic resources available for most species (Ainouche *et al.*, 2009;
107 Richards *et al.*, 2017). Increasing application of high-resolution methods to understand genome-
108 level response to environmental conditions in natural populations is one of the central current
109 tasks of ecological genomics.

110 In 2010, the *Deepwater Horizon* (*DWH*) oil spill developed into the largest marine oil
111 spill in history (National Commission on the BP *Deepwater Horizon* oil spill, 2011), and became
112 an opportunity to apply emerging genomics technologies in natural populations to examine
113 responses to this recurrent anthropogenic stress in a diversity of organisms (e.g. Hazen *et al*.
114 2010; Whitehead *et al*. 2012; Kimes *et al*. 2013, 2014; Alvarez *et al.* 2018; DeLeo *et al.* 2018). A
115 mixture of crude oil made and dispersants made landfall along 1,773 kilometers on the shorelines
116 of Louisiana, Mississippi, Alabama and Florida (Mendelssohn *et al.*, 2012; Michel *et al.,* 2013).
117 Nearly half of the habitat affected was salt marsh, which provides valuable ecosystem functions
118 such as providing nurseries for birds and fish, and buffering storm and wave action (Day *et al.*,
119 2007; Mendelssohn *et al.*, 2012; Michel *et al.,* 2013). Gulf of Mexico salt marshes are dominated
120 by the foundation species *Spartina alterniflora,* which is remarkably resilient to a variety of
121 environmental stressors (Pennings & Bertness, 2001; Baisakh *et al.*, 2008; Baisakh & Subudhi,
122 2009; Silliman *et al.*, 2012; Bedre *et al.*, 2016). Crude oil exposure from the *DWH* oil spill
123 resulted in reduced carbon fixation, reduced transpiration, and extensive above-ground dieback
124 in *S. alterniflora* populations (Lin & Mendelssohn, 2012; Silliman *et al.,* 2012), but oil-affected
125 populations showed partial to complete recovery within seven months of the spill (Lin *et al.,*
126 2016). However, the genomic and regulatory mechanisms that underlie this remarkable recovery
127 have been poorly characterized.

128 Despite the tremendous resilience of *S. alterniflora* to the *DWH* spill, we discovered
129 genetic divergence of individuals from oil-exposed areas and nearby uncontaminated areas
130 (Robertson *et al.*, 2017). We also found that pollution tolerance may be modulated by a diverse
131 set of candidate genes (Alvarez *et al.*, 2018). In particular, we provided support for the role of an
132 epigenetic regulator (a homolog of the histone methyltransferase SUVH5), which significantly
133 altered growth and flower production under crude oil stress in experimental studies (Alvarez *et*
134 *al.*, 2018). Considering that histone modifications may regulate and be regulated by DNA
135 methylation (reviewed in Du *et al.*, 2015), we expected to find a strong correlation between
136 patterns of DNA methylation and crude oil exposure. However, while a few DNA methylation

137 loci (measured via methylation sensitive amplified fragment length polymorphism; MS-AFLP)
138 were correlated with oil exposure, we did not find genome-wide patterns in DNA methylation
139 correlated with oil exposure in *S. alterniflora* (Robertson *et al.*, 2017). Our findings supported
140 genetic differentiation in response to exposure to crude oil, but suggested that our ability to
141 detect changes in DNA methylation may have been limited by the number of MS-AFLP markers
142 (39 polymorphic loci). Further, although our previous study identified population-level patterns
143 of divergence, we were unable to associate the anonymous AFLP loci with gene function
144 (Schrey *et al.*, 2013).
145 In this study, we used a recently developed reduced representation bisulfite sequencing
146 technique, epigenotyping by sequencing (epiGBS), to generate a more robust DNA sequence and
147 DNA methylation data set (van Gurp *et al.*, 2016). We expected that the increased resolution,
148 both in number and in detail of the markers, provided by this sequencing approach would allow
149 us to identify fine scale DNA methylation structure that was not apparent in our previous MS-
150 AFLP study. We further expected that biasing toward coding regions of the genome with the use
151 of methylation sensitive enzymes in the epiGBS protocol (van Gurp *et al.*, 2016) would allow us
152 to find evidence that DNA methylation was correlated with changes in gene function since some
153 fragments might overlap with the promoter or 5' end of the coding regions of genes (Niederhuth
154 & Bewick *et al.*, 2016). Therefore, we hypothesized that genome-wide expression, as well as
155 expression of candidate genes, could be correlated to changes in genetic variation and DNA
156 methylation.
157
158 **Materials and Methods**
159
160 *Sample Collection*
161 We collected individuals from the leading edge of the marsh at three contaminated and
162 three uncontaminated sites near Grand Isle, Louisiana and Bay St. Louis, Mississippi in August
163 2010, four months after the *DWH* oil spill as described in previous studies (Table 1; Robertson *et*
164 *al.,* 2017; Alvarez *et al.,* 2018). These sites were naturally highly variable in conditions, but all
165 sites supported monocultures of *S. alterniflora*. Contaminated sites were identified by the visual
166 presence of oil on the sediment, and substantial above-ground dieback of *S. alterniflora* on the
167 leading edge of the marsh with *S. alterniflora* plants growing up through the dead wrack. Nearby

168 uncontaminated sites did not have any visual signs of the presence of oil or noticeable dieback of
169 the above ground portions of *S. alterniflora* (contamination status confirmed via NRDA
170 databases, Robertson *et al.*, 2017). To standardize age and minimize developmental bias in
171 sampling, we collected the third fully expanded leaf from each of eight individuals, spaced 10
172 meters apart at each of the six sites (N=48). Leaf samples were immediately frozen in liquid
173 nitrogen to prevent degradation, and kept frozen during transport to the University of South
174 Florida for processing and analysis.
175
176 *DNA extractions and library prep*
177 We isolated DNA from each sample (48) using the Qiagen DNeasy plant mini kit
178 according to the manufacturer's protocol. We prepared epiGBS libraries *sensu* van Gurp *et al.*
179 (2016). Briefly, isolated DNA was fragmented with the enzyme PstI, which is sensitive to CHG
180 methylation and biases resulting libraries toward coding regions (van Gurp *et al.,* 2016). After
181 digestion, adapters with variable barcodes were ligated to either end of the resulting fragments.
182 Adapters contained methylated cytosines to ensure their sequence fidelity through the subsequent
183 bisulfite treatment. We used the Zymo EZ Lightning methylation kit to bisulfite treat and clean
184 the DNA. Libraries were then amplified with the KAPA Uracil Hotstart Ready Mix with the
185 following PCR conditions: an initial denaturation step at 98ºC for 1 min followed by 16 cycles of
186 98ºC for 15s, 60ºC for 30s, and 72ºC for 30s, with a final extension of 72ºC for 5 min. Paired-
187 end reads were sequenced at the University of Florida Interdisciplinary Center for Biotechnology
188 Research on the Illumina HiSeq 5000.
189
190 *Data pre-processing and mapping to transcriptome*
191 We used the epiGBS pipeline (van Gurp *et al.,* 2016) to demultiplex samples, trim
192 adapter sequences, and assemble the *de novo* reference sequence
193 (https://github.com/thomasvangurp/epiGBS). To improve variant calling while accounting for
194 polyploidy, we realigned individuals to the *de novo* reference (generated from the epiGBS
195 pipeline), separately using BWAmeth (Pedersen *et al.,* 2014), and called variants using the
196 GATK pipeline (DePristo *et al.,* 2011) with a specified ploidy of six. Methylation was tabulated
197 using the existing epiGBS analysis pipeline. Both SNP and methylation data were filtered
198 separately to include only loci that were present in more than 90% of individuals, with no more

199 than 50% missing from any one individual. Thus, each locus had, at most, 10% missing data,
200 while each individual had less than 50% missing data across all loci. We removed loci with less
201 than 5x depth of coverage and remaining missing data were imputed via a k-nearest neighbors
202 approach, yielding a matrix of "common loci" (impute, Hastie *et al.*, 2018).
203 All fragments were mapped to the published *S. alterniflora* transcriptome (Boutte *et al.,*
204 2016) and the *Oryza sativa* genome (Michigan State University version 7, Kawahara *et al.*, 2013)
205 using BLAST (Altschul *et al.,* 1997). We estimated the number and order of exons by mapping
206 the *S. alterniflora* transcriptome to *Oryza sativa*. We also tested for random sampling across
207 exons among the epiGBS fragments that occurred within coding regions using a Kolmogorov-
208 Smirnov test. We used BLAST (Altschul *et al.,* 1997) and RepeatExplorer (Novak *et al.,* 2013)
209 to compare our sequenced fragments to the *S. alterniflora* transcriptome (Ferreira de Carvalho *et*
210 *al.,* 2013, 2017; Boutte *et al.*, 2016) and known repeat elements, respectively.
211
212 *Population genetics*
213 Our RRBS did not provide sufficient sequencing depth to estimate hexaploid genotype
214 likelihoods with confidence, particularly considering the high levels of heterozygosity measured
215 in *S. alterniflora* populations (Richards *et al.*, 2004; Travis & Hester, 2005) and the lack of a
216 high-quality reference genome (Dufresne *et al.,* 2014; Boutte *et al.,* 2016). We therefore used the
217 frequency of the most common allele within an individual at each polymorphic locus to estimate
218 allelic diversity, and to estimate Nei's genetic distances among individuals and sites (StAMPP,
219 Pembleton *et al.,* 2013).
220 We obtained pairwise F_{ST} values between populations to test for significance of pairwise
221 differences. Following recommendations from Meirmans (2015) to minimize false positives, we
222 used distance-based redundancy analysis (RDA; Vegan, Oksanen *et al.* 2017) to assess isolation
223 by distance by regressing the genetic relatedness matrix against latitude and longitude (genetic
224 distance ~ latitude * longitude) for each site using constrained analysis of principal coordinates
225 (Vegan, Oksanen *et al.,* 2017).
226 To quantify the relationship between genome-wide variation and environmental
227 conditions, we used RDA with surrogate variables to represent the complex differences among
228 sites with the formula (genetic distance ~ oil exposure + surrogate variables). Redundancy
229 analysis allowed us to assess the joint influence of all SNPs simultaneously (Lasky *et al.*, 2015;

230 Forester et al. 2016). Although we attempted to control for variation among samples and sites
231 with a replicated sampling strategy, rather than using a single term for "site", we included
232 parameters identified by surrogate variable analysis (SVA), which provides a method to detect
233 residual variation due to unmeasured differences among populations including environmental
234 variation, life history variation, and geographical separation, between samples that may distort
235 analyses (Leek *et al.*, 2017). We estimated surrogate variables by fitting a full and null model to
236 the appropriate (i.e. DNA sequence allele frequency or DNA methylation frequency) high
237 dimensional data matrix and examining the principal components of the residuals (Leek &
238 Storey, 2007). We used a permutational test (999 permutations) to assess the significance of
239 differences between the treatment and control groups, given the covariates. We also tested for
240 differences in the amount of dispersion around centroids (multivariate variance) between oil-
241 exposed or unexposed populations (PERMDISP2, Vegan, Oksanen *et al.* 2017). We corrected
242 the resulting P values for false discovery using a Holm correction, and visualized results using
243 multidimensional scaling (MDS, Vegan, Oksanen *et al.* 2017).
244 To identify SNPs that were significantly correlated with oil contamination, we used
245 binomial linear mixed modeling (MACAU; Lea *et al.,* 2015) including a genetic relatedness
246 matrix to account for population structure and the surrogate variables as covariates. All locus-by-
247 locus association scans were corrected for family-wise false discovery using the Q-value package
248 in R (Storey *et al.,* 2015).
249
250 *Methylation analysis*
251 During the filtering process, loci were annotated with their methylation context, but all
252 contexts were pooled for distance-based analyses as well as family-wise error rate corrections
253 after locus-by-locus modeling. We tabulated methylation frequency at each locus, defined as the
254 fraction of methylated cytosines observed out of the total number of cytosines measured at a
255 given locus (methylated cytosines/(methylated+unmethylated cytosines)), and used methylation
256 frequencies to generate a methylation distance matrix in R.
257 To identify signatures of DNA methylation variation that were correlated with oil
258 exposure and not genetic structure, we used principal components analysis to reduce the genetic
259 data to orthogonal components, and identified 8 of the 42 possible principal components axes
260 that were significantly correlated with methylation. We then modeled the impact of oil exposure

261 to genome-wide patterns of DNA methylation by jointly assessing the influence of all
262 polymorphic methylation loci concurrently via RDA (Vegan, Oksanen *et al.* 2017) with the
263 formula (methylation distance ~ oil exposure + surrogate variables + significant principal
264 components of allele frequency data). We corrected the resulting P values for false discovery
265 using a Holm correction, and visualized results using multidimensional scaling (MDS, Vegan,
266 Oksanen *et al.* 2017).
267 To identify differentially methylated positions (DMPs) between contaminated and
268 uncontaminated samples, we used binomial linear mixed modeling (MACAU; Lea *et al.,* 2015),
269 using the genetic relatedness matrix and surrogate variables as covariates to control for
270 population structure. We corrected locus-specific P-values for multiple testing (qvalue, Storey *et*
271 *al.,* 2015), and tested for overrepresentation of cytosine contexts (CG, CHG, CHH) using an
272 exact binomial test, implemented in R. Because our epiGBS fragments rarely exceeded 200bp,
273 we did not identify differentially methylated regions.
274
275 *Relationships to gene expression variation*
276 We used transcriptome data from our previous analyses of three mRNA pools of three
277 individuals from each of the six sites to assess the relationship between variation in gene
278 expression, genetic variation, and methylation variation (Alvarez *et al.*, 2018). We found 3,334
279 genes that responded to crude oil exposure in *S. alterniflora* out of 15,950 genes that were
280 assessed. In order to make the epiGBS data comparable to our pools of mRNA, we concatenated
281 SNPs and methylation polymorphisms from individuals into *in silico* sample pools by averaging
282 values at individual loci across samples within pools of the same individuals that were used in
283 the gene expression analysis. We then calculated genetic, expression, and methylation distances
284 between sample pools and used multiple regression on distance matrices to assess the
285 relationship between all three data types (MRM; ecodist, Goslee & Urban, 2007).
286 We assessed the correlation between gene expression, genetic variation, and methylation
287 variation within the same fragment (i.e. in *cis*) gene-by-gene using only microarray probes and
288 epiGBS fragments that could be mapped to the *S. alterniflora* transcriptome. We regressed the
289 expression values of each gene against the methylation frequencies and most common allele
290 frequencies at corresponding epiGBS fragments using the *lm* function in R with the formula
291 (gene expression ~ *cis* methylation + *cis* SNP). We summarized variation across each epiGBS

292 fragment for multiple SNP or methylation loci by using the first principal component of each
293 data type. We interpreted significant effects of SNPs or DNA methylation to mean that the
294 expression of a given gene depended on the frequency of the nucleotide or methylation
295 polymorphism. We corrected for false discovery using qvalue (Storey *et al.*, 2015).
296
297 **Results**
298 *epiGBS yields informative genetic and methylation loci*
299 Following library preparation, we removed four samples due to stochastic under-
300 sequencing. The libraries for 44 individuals (Table 1) generated 6,809,826 total raw sequencing
301 reads, of which 3,833,653 (56.3%) could be matched to their original mate strand. *De novo*
302 assembly using the epiGBS pipeline resulted in 36,131 contiguous fragments (19-202 basepairs)
303 with a total length of 5,441,437 bp (Figs. **S1, S2**). Given the size of the *S. alterniflora* genome
304 (2C values = 4.3 pg; Fortune *et al.*, 2008), our epiGBS approach assayed approximately 0.3% of
305 the genome. However, fragments that were >90% similar were merged, and polyploid
306 homeologs may have been concatenated. With BLAST, we found 10,103 fragments mapped to
307 2,718 transcripts in *S. alterniflora* transcriptome. We found no bias in mapping to particular gene
308 regions (Fig. **S3**), but 1,571 transcripts (57.8%) contained multiple epiGBS fragments that align
309 to the same place, and 296 (10.9%) contained multiple epiGBS fragments that mapped to
310 different places within the same gene. We suspect that multiple epiGBS fragments map to the
311 same location because some epiGBS contiguous fragments represent different homeologs of the
312 same region, which mapped to the same location. Only 1% of reads map to repetitive elements,
313 confirming that *Pst1*-fragmented libraries were biased away from highly methylated, repetitive
314 regions (van Gurp *et al.,* 2016).
315
316 *Genetic differentiation*
317 Our initial sequencing run yielded 171,205 SNPs across all individuals. After filtering to
318 common loci and removing invariant sites, we imputed 10-25% missing genetic data and 10-50%
319 methylation data per sample (Fig. **S4**), resulting in 6,521 SNP loci. We hypothesize that this
320 significant data loss after filtering was due to insufficient sampling depth across an exceptionally
321 large hexaploid genome, and a preponderance of restriction sites. Of these 6,521 loci, 243 SNPs
322 occurred in transcripts. Pairwise F_{ST} calculations showed that all sites were significantly different

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

354 experienced frequency changes of 30%, and was located in an actin filament depolymerization
355 protein (Os03g56790; ADF2). We found no DMPs that mapped to the first 250 nucleotides of a
356 fragment in exon 1, where DNA methylation may be particularly relevant to the differential
357 expression of genes (Secco *et al.*, 2015).
358
359 *Correlations with gene expression*
360 Although 2,718 epiGBS fragments mapped to the *Spartina* transcriptome, none of these
361 fragments overlapped with transcripts that showed expression differences correlated with
362 exposure to the *DWH* oil spill (Alvarez *et al.*, 2018). Across all genes within a sample, we found
363 no significant relationship between genetic distance with gene expression distance (P=0.185,
364 Mantel's R=0.1489), but there was a correlation between patterns of methylation variation and
365 genome wide gene expression (P=0.009, Mantel's R=0.2319). We had expression data and either
366 SNPs or methylation polymorphisms for only 54 genes, and we found no association between
367 individual SNPs or methylation polymorphisms with expression in *cis*.
368
369 **Discussion**
370 *Spartina alterniflora* displays high levels of genetic and methylation variation across
371 environmental conditions in its native range (Richards *et al.*, 2004; Hughes & Lotterhos, 2014;
372 Foust *et al.,* 2016; Robertson *et al.*, 2017), providing substrate for both genetic and epigenetic
373 response to pollution. We previously found that genetic structure and expression of 3,334 genes
374 were correlated to exposure to the *DWH* oil spill, but genome-wide methylation variation was
375 not (Robertson *et al.,* 2017; Alvarez *et al.*, 2018). Higher resolution epiGBS suggests that both
376 genetic sequence and DNA methylation are correlated with crude oil exposure in *S. alterniflora*,
377 and that changes in gene expression are more strongly correlated to genome-wide patterns of
378 DNA methylation than population structure.
379
380 *Increased resolution of genetic and epigenetic response to the DWH*
381 Our study confirmed significant genetic differentiation between oil-exposed and
382 unexposed sites. This type of genetic structure may reflect either stochastic mortality in oil-
383 exposed areas that may result from a severe bottleneck, or a signature of selection for oil
384 tolerance in affected populations. *Spartina alterniflora* is a highly stress tolerant plant, and

385 populations have persisted after exposure to the *DWH* oil spill, even after extensive dieback (Lin
386 & Mendelssohn, 2012; Silliman *et al.*, 2012; Lin *et al.*, 2016). However, our genetic data suggest
387 that at least some *S. alterniflora* genotypes were susceptible to crude oil stress, and either had not
388 regrown at the time of sampling or experienced mortality as a result of oil exposure. Our genetic
389 marker studies, combined with initial losses in live aboveground and belowground biomass,
390 support the hypothesis of differential mortality (Lin *et al*., 2016). Although we found no
391 evidence for a reduction in genetic variation, which may indicate selection for tolerant
392 genotypes, the polyploidy of *Spartina* makes accurate quantification of total genetic variation
393 challenging. Further investigations will be required to confirm whether mortality varied by
394 genotype, and whether the *DWH* oil spill was truly a selective event.
395 The DNA methylation differences that were correlated to oil exposure may reflect either
396 the downstream effects of different genetic variants, a plastic regulatory response to oil exposure,
397 or both. In another study of *S. alterniflora* populations, patterns of DNA methylation were more
398 strongly correlated than genetic structure with microhabitat, and some response of DNA
399 methylation to environment was independent of population structure (Foust *et al.,* 2016). In the
400 oil contaminated populations, we identified a differentially expressed gene that was homologous
401 to the histone methyltransferase SUVH5, which may modulate fitness effects during oil exposure
402 (Alvarez *et al.,* 2018). Histone methylation is intimately linked to DNA methylation through the
403 regulation of *DNA (cytosine-5)-methyltransferase CMT3* activity (Stroud *et al.*, 2013). Therefore,
404 we hypothesized that the differential expression of SUVH5 in response to crude oil exposure
405 would result in differences in DNA methylation variation. With the increase in resolution offered
406 by the epiGBS data set (36,131 epiGBS fragments yielding 19,615 methylation loci compared to
407 39 polymorphic MS-AFLP fragments), we found a previously unidentified genome-wide
408 signature of methylation differentiation between oil affected and unaffected sites after
409 controlling for population structure.
410
411 *Epigenetic variation is correlated to genome wide patterns of gene expression*
412 We expected that our fragments would be biased toward coding regions of the genome,
413 and that we would find DNA methylation was correlated with changes in gene function since
414 fragments could overlap with the promoters or the 5' end of the coding regions of genes. We
415 found that almost 1/3 (10,103 of the 36,131) epiGBS fragments mapped to the *S. alterniflora*

416 transcriptome. However, many transcripts contained multiple epiGBS fragments such that only
417 2,718 transcripts were represented. None of these 2,718 genes overlapped with the 3,334 oil
418 responsive genes from our previous transcriptome analysis (Alvarez *et al.*, 2018), and few
419 fragments overlapped the 5' end of genes. Therefore, our expectation that we could correlate
420 local methylation polymorphisms to expression of oil responsive genes was not fulfilled in this
421 study. However, genome-wide patterns of DNA methylation are more strongly correlated than
422 genetic structure with gene expression.
423 Combined, our results suggest that genetic variation and regulatory mechanisms may play
424 a role in the response of *S. alterniflora* populations to crude oil pollution. Although we are
425 unable to dissect causality, our data suggest one of two possibilities. In the first scenario, crude
426 oil exposure may have generated genetic differentiation between oil exposed and unexposed
427 populations, which in turn shaped patterns of gene expression. Differential transcription may
428 then have generated divergent patterns of methylation, either through genetic variation in DNA
429 methyltransferases (Dubin *et al.*, 2015), or through changes in gene expression affected by
430 neighboring transposable element activity (*sensu* Secco *et al.*, 2015). In this scenario, DNA
431 methylation patterns are generated by genetic variation and/or reflect expression variation (*sensu*
432 Meng *et al.,* 2016; Niederhuth & Schmitz, 2017). In the second scenario, crude oil exposure may
433 have affected genetic variation while simultaneously but independently inducing patterns of
434 DNA methylation e.g. as a response to the genomic "shock" of crude oil exposure (Rapp &
435 Wendel, 2005; Richards *et al.*, 2012b). This variation may modulate the plastic response of gene
436 expression variation in this system, and reflect an induced regulatory shift in *S. alterniflora*,
437 regardless of genetic background. Of course, these scenarios are not mutually exclusive. While
438 more complex models of causation will require substantially more genomic resources than are
439 currently available in *S. alterniflora*, reverse genetic screens and higher resolution surveys may
440 help to discern the relative contributions and causal roles of methylation and genetic variation in
441 genes of interest.
442
443 *Reduced representation sequencing compared to AFLP*
444 As the field of ecological genomics matures, there is a pressing need to develop robust
445 assays and statistically sound measures of population regulatory variation. Reduced
446 representation methylation sequencing techniques are attractive for ecological genomics because

447 they can be used to infer genome wide patterns of both genetic and methylation variation without
448 a high-quality reference genome (Robertson & Richards, 2015; Richards *et al.*, 2017; Paun *et al.*,
449 2018). However, considering the small portion of the genome that is sampled in these
450 techniques, they still have serious limitations particularly for species that do not yet have a fully
451 sequenced reference genome (Paun *et al.*, 2018).

452 We assessed the utility of epiGBS compared to MS-AFLP, and expected that the
453 substantial increase in informative markers would confirm our results from our MS-AFLP study
454 and lend greater resolution to detect patterns of DNA methylation variation. Our epiGBS survey
455 detected significant differentiation in both genetic variation and DNA methylation that was
456 correlated to oil exposure, suggesting that epiGBS provides increased resolution over MS-AFLP
457 to detect genome-wide methylation differences in studies of similar sample sizes. However,
458 despite the much larger data set generated by epiGBS compared to MS-AFLP, we only found 14
459 differentially methylated positions, which is not substantial improvement over the five loci
460 detected by MS-AFLP (Robertson *et al.*, 2017). The comparable number of DMPs and lack of
461 power to evaluate *cis* association between methylation, genetic variation, and gene expression
462 suggests that the ability to characterize individual functional elements remains obscured by
463 substantial variation in methylation across individuals, and is limited by the small fraction (an
464 estimated 0.3%) of the genome that is assayed. Further, although we may have biased sampling
465 to coding regions of the genome, we found few fragments that overlapped the functionally
466 relevant 5' end of transcripts. Without a reference genome for *S. alterniflora,* we were unable to
467 identify fragments that overlapped promoter regions.

468 Sequencing-based techniques provide the potential to identify functional genomic
469 regions, but correct annotations rely on genomic resources in a relevant reference. In polyploid
470 species like *S. alterniflora*, a greater number of paralogs and the potential for
471 neofunctionalization among them creates additional uncertainty for using annotations from other
472 species (Primmer *et al.*, 2013). *Spartina alterniflora* has various levels of duplicated gene
473 retention and homeologous expression (Ainouche *et al.*, 2003; Fortune *et al.*, 2007; Ferreira de
474 Carvalho, 2013, 2017; Boutte *et al.*, 2016), which may result in more opportunities for gene
475 diversification and subfunctionalization (Chen *et al.*, 2015; Shimizu-Inatsugi *et al.* 2017), and
476 complicates gene annotation. Therefore, while studies with RRBS techniques in non-model
477 plants offer increased power to detect broad, genome-wide patterns of variation that may be

478 correlated to ecology, they are still limited for the detection of specific gene function. Future
479 studies will benefit from optimizing protocols that enrich for specific portions of the genome, but
480 generating a draft reference genome will be imperative to locate the promoter regions and allow
481 for better exploitation of RRBS data (Paun *et al.*, 2018).
482

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491 **Tables and Figures**

492

493 **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

494

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

496 **Table 2** Association between oil exposure, genetic distance, and methylation distance across
497 tests.

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

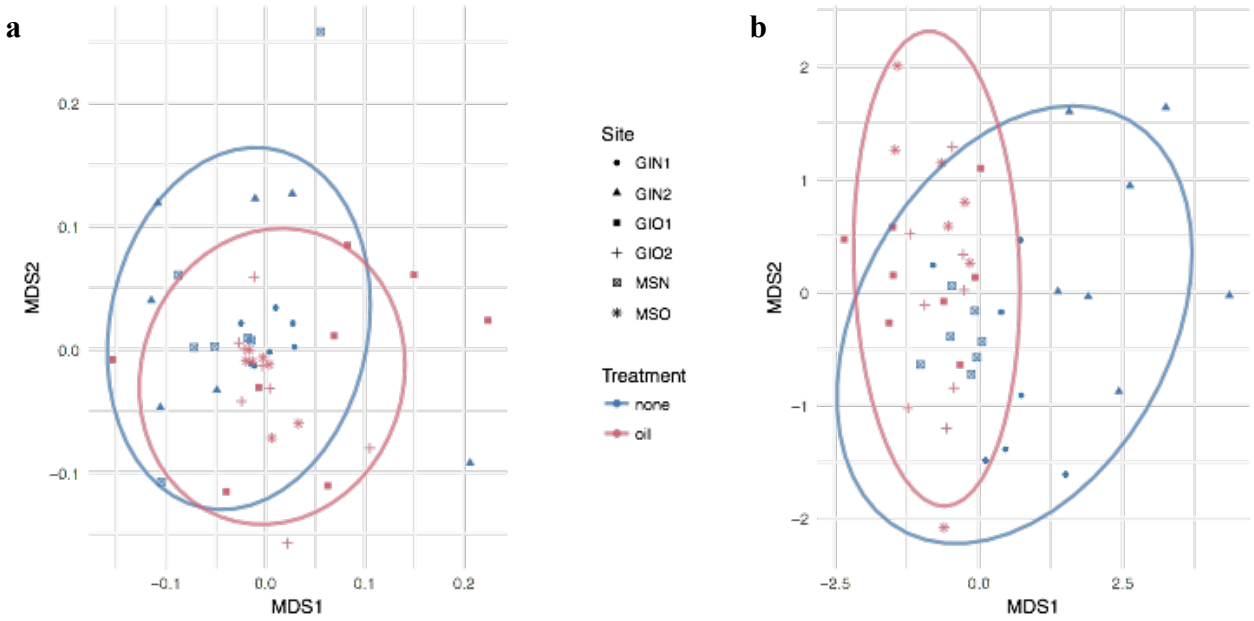
498

499 Test statistics and significance, determined through RDA. For clarity, we do not show
500 significance for estimated surrogate variables (7 for methylation data, 16 for genetic data) or the
501 8 principal components of allele frequencies for the methylation data. P * ≤ 0.05, P ** ≤ 0.01, P
502 *** ≤ 0.0001

503 **Table 3** Pairwise Fst among three oil contaminated and three uncontaminated sites. Bold (i.e. all
504 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**

505



506 **Figure 1** Multidimensional scaling (MDS) of **a**) genetic distance matrix and **b**) DNA
507 methylation distance matrix. Circles represent 95% confidence intervals around the centroid of
508 each group: no oil exposure ("none") or oil-exposed ("oil").
509

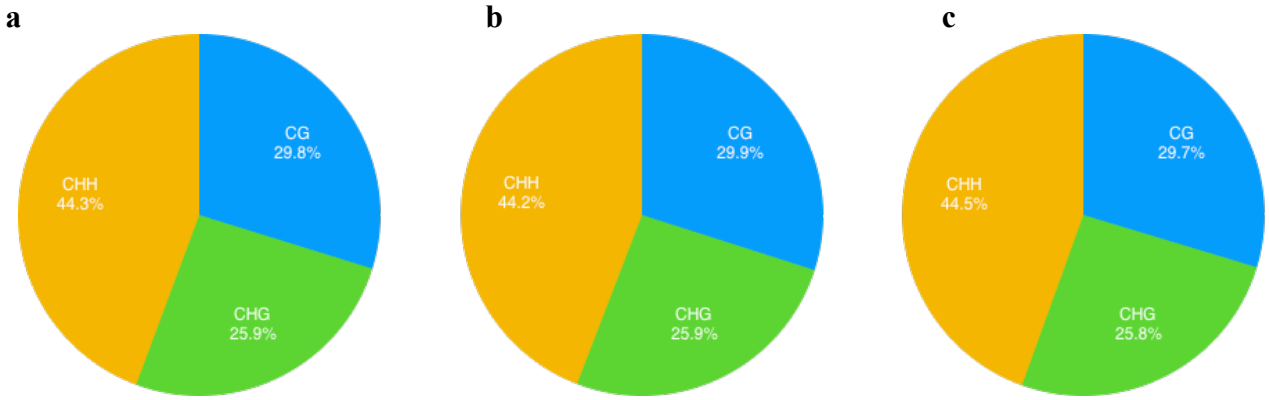


Figure 2 Proportion of methylated sites by context across a) all samples, b) in uncontaminated sites and c) oil-contaminated sites.

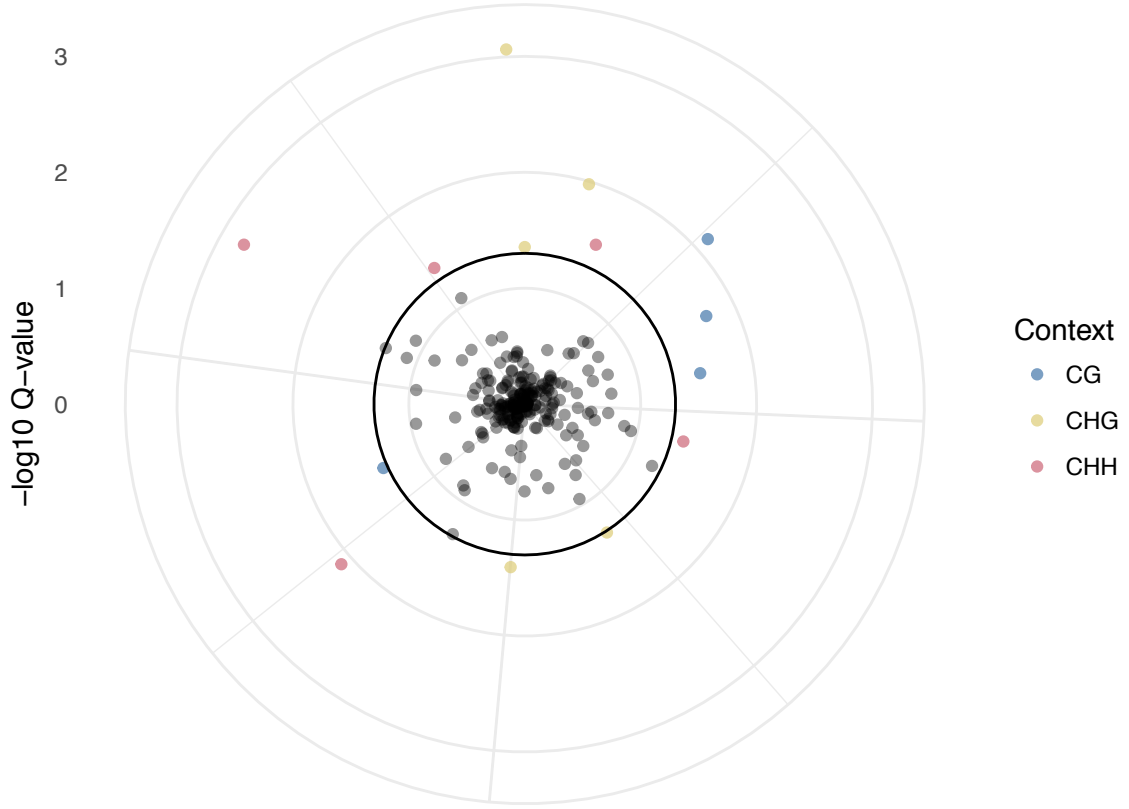


Figure 3 Circular manhattan plot showing 14 significant outlier methylation loci, as determined by Q-value. Only significant loci (Q<0.05) are colored, where colors represent trinucleotide context. Plot is shown as circular to accommodate unknown positions along a chromosome, as *S. alterniflora* does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

523 **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		

524

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

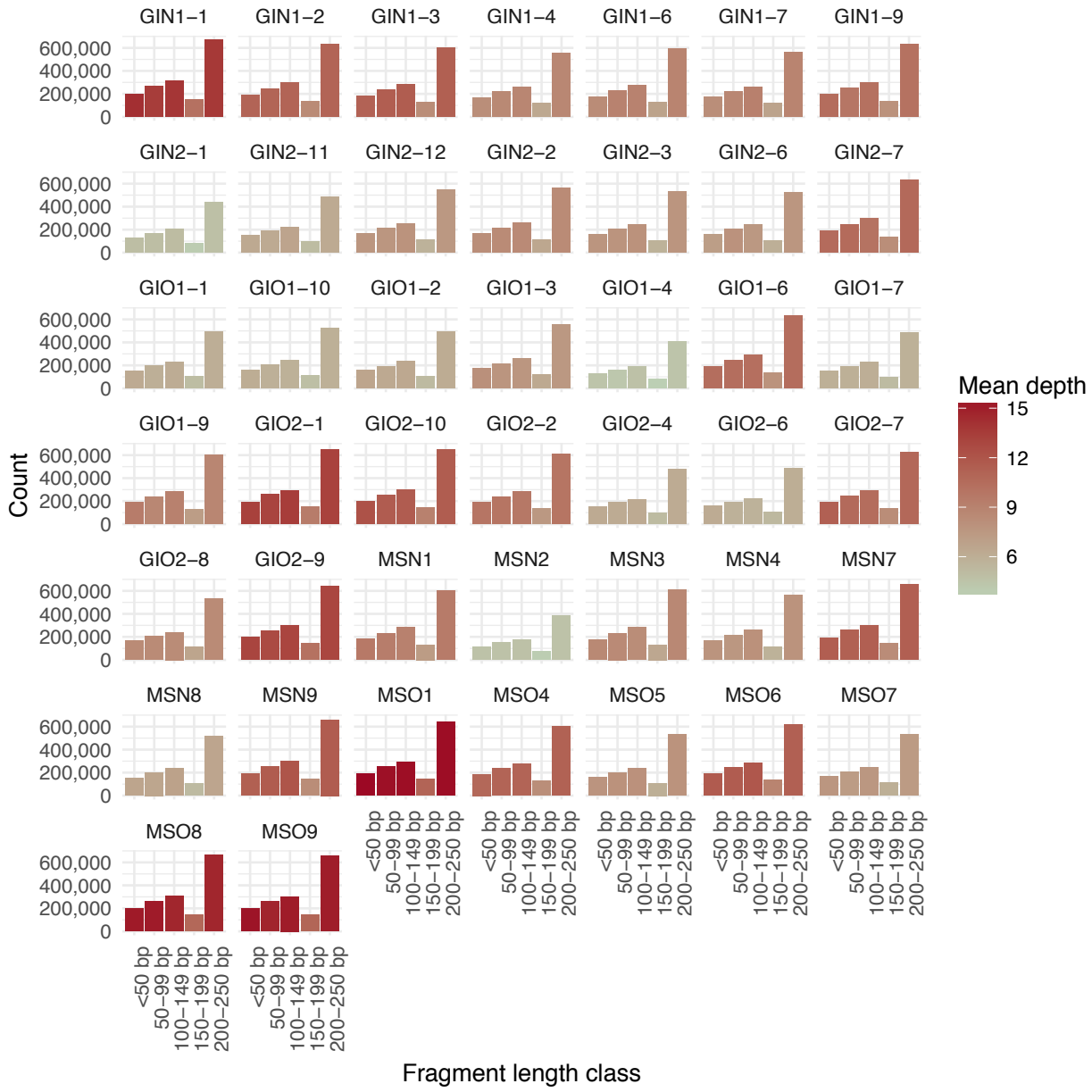


Figure S1 Depth of sampling by fragment size in each sample across all fragments. Fragments ranged from 19-202 basepairs (bp) for a total length of 5,441,437 bp (approximately 0.3% of the 2117 Mbp *S. alterniflora* genome (2C= 4.33pg); Fortune *et al.,* 2008). Fragments shown in classes on the X axis.

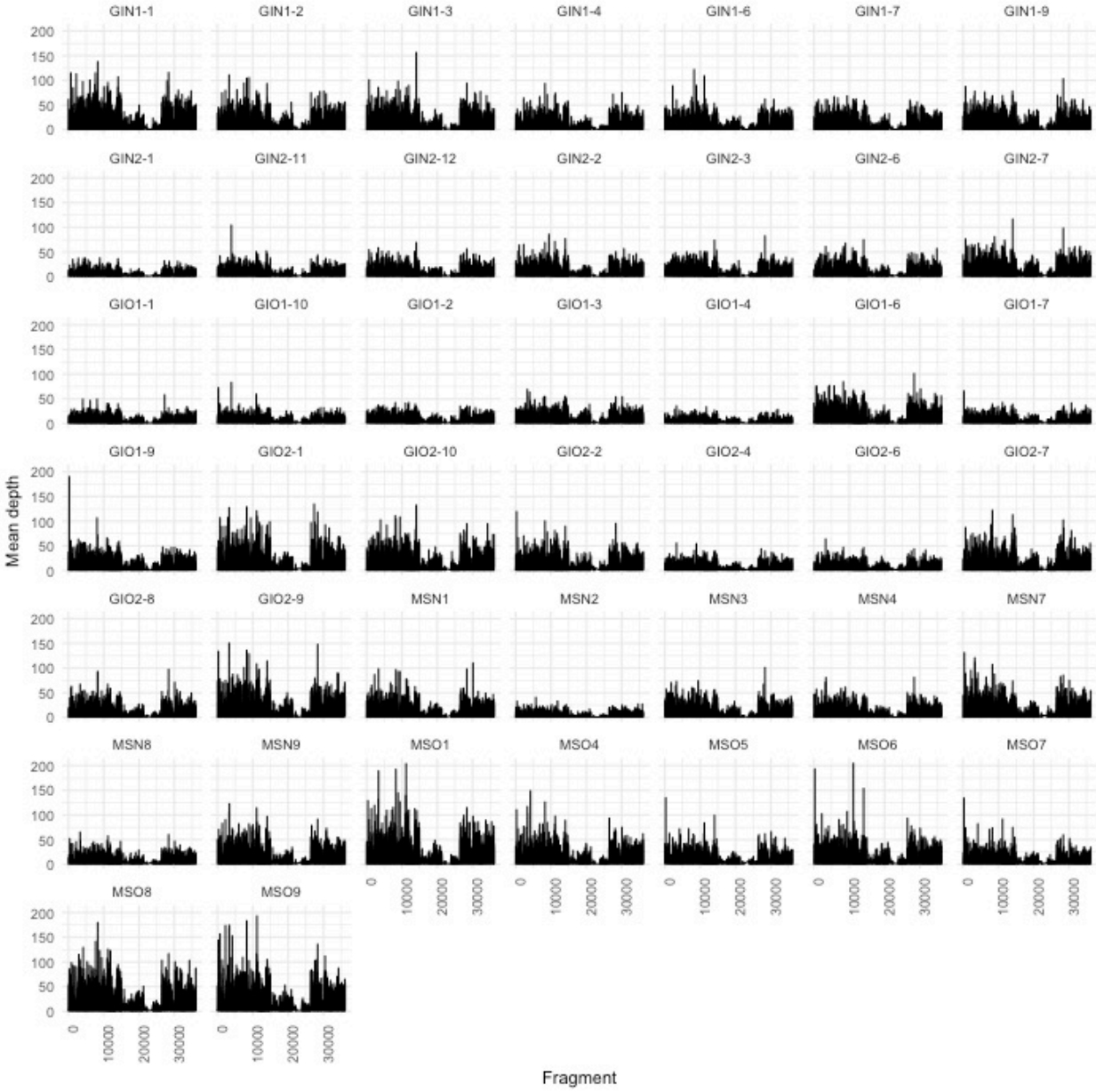
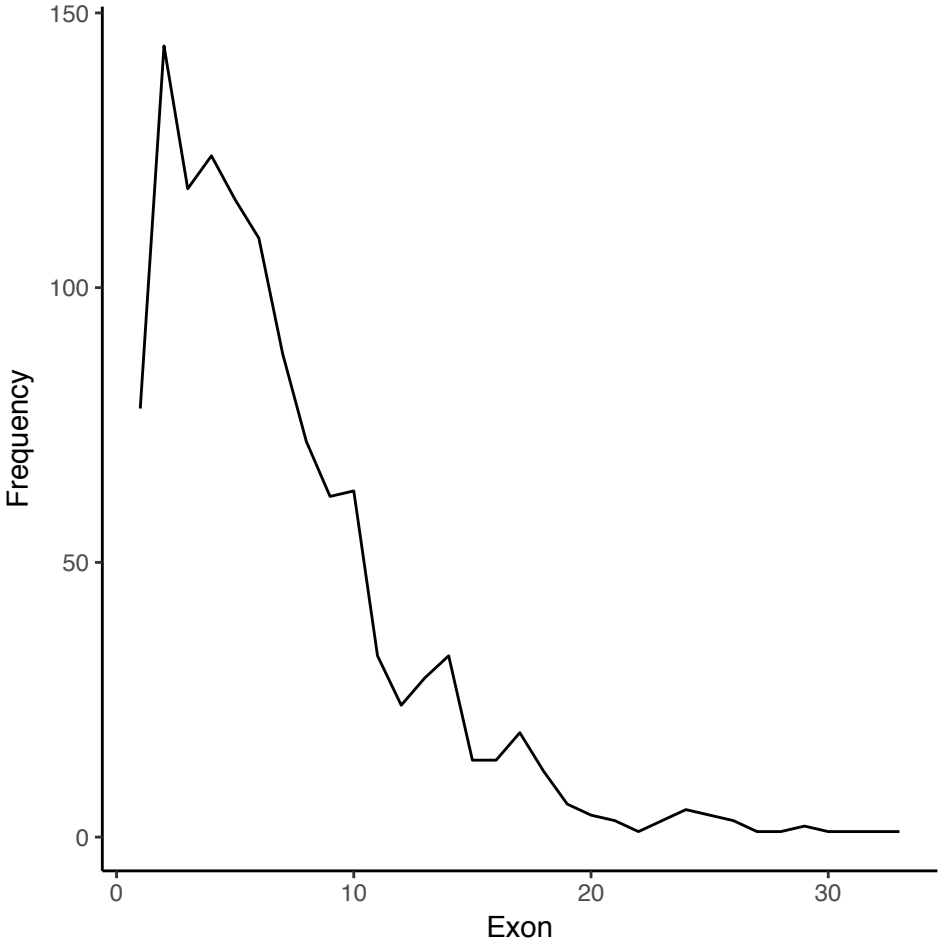


Figure S2 Sequencing depth for each sample for each of the 36,131 fragments.



540
541 **Figure S3.** Distribution of epiGBS hits across exons. Exon order is predicted by BLAST hits to
542 *Oryza sativa*.
543

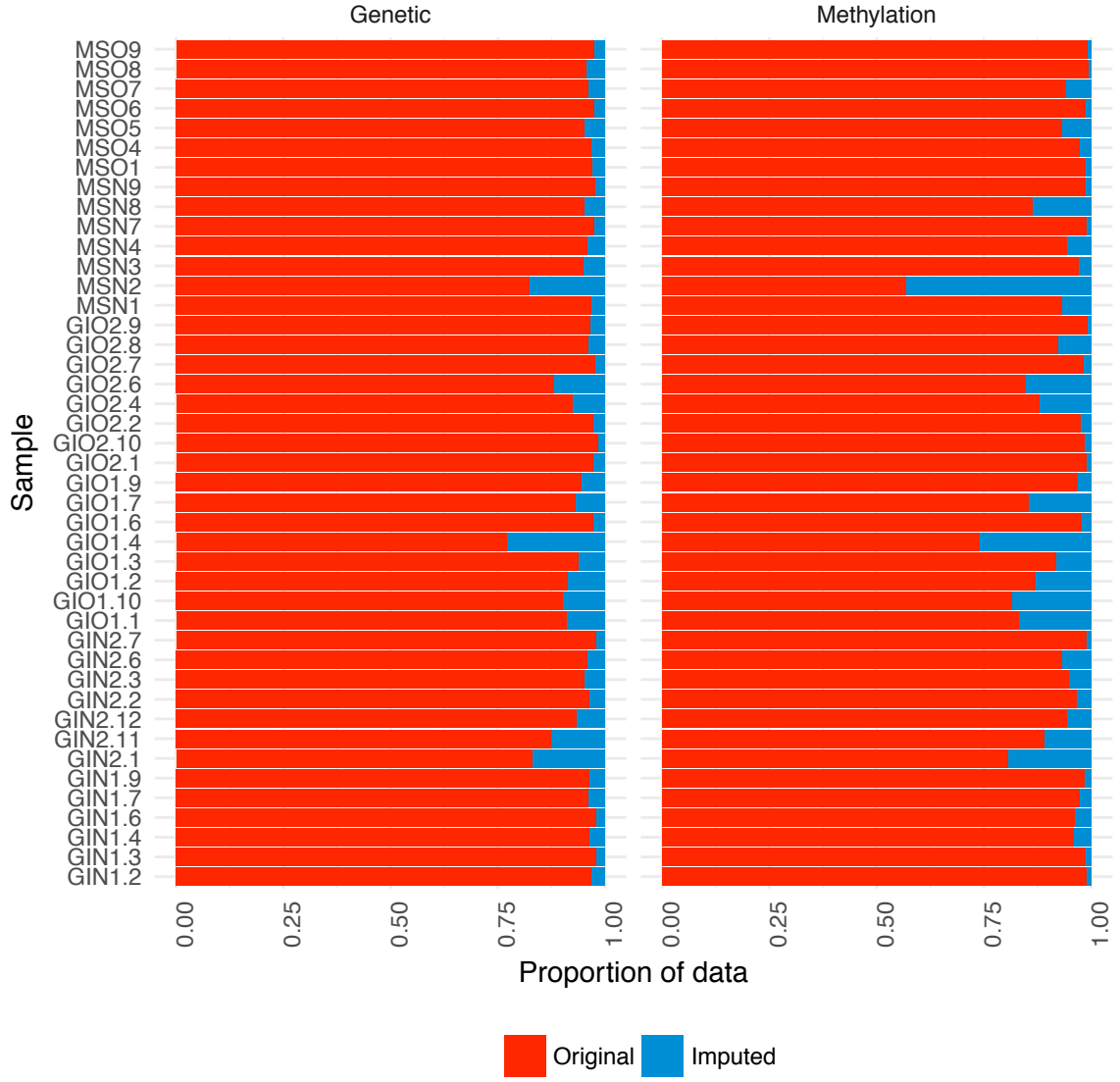


Figure S4. Missing and imputed data by sample after filtering. Genetic and methylation data were both filtered to include only loci present in >90% of samples and samples with less than 50% missing data.

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