Reduced representation characterization of genetic and epigenetic differentiation to oil pollution in the foundation plant *Spartina alterniflora*

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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 documented genetic and methylation differentiation between oil-exposed and unexposed populations, suggesting that the *Deepwater Horizon* oil spill may have selected on genetic variation, and either selected on epigenetic variation or induced particular epigenotypes and expression patterns in exposed compared to unexposed populations. In support of the potential for differential response to the *Deepwater Horizon* oil spill, we demonstrate genotypic differences in response to oil under controlled conditions. Overall, these findings demonstrate genetic variation, epigenetic variation and gene expression are correlated to exposure to oil pollution, which may all contribute to the response to environmental stress.

68 **Introduction**

69 Organismal interactions and response to environment are governed by molecular
70 mechanisms, which are among the most basic levels of biological organization. Studies across a
71 diversity of organisms have described the association of genetic variation with environmental
72 factors (Andrew *et al.*, 2013; Feder & Mitchell-Olds, 2003). More recently, transcriptomics
73 studies in natural populations have identified gene expression differences that are associated with
74 phenotypic plasticity, genotype-by-environment interactions, and local adaptation, and that some
75 of these differences are only elicited in natural environments (Alvarez, Schrey, & Richards,
76 2015; Nagano *et al*. 2012, 2019; Nicotra *et al.*, 2010). Hence, gene expression variation, like
77 genetic variation, can translate into trait variation that contributes to organismal performance
78 with important population- and community-level ecological effects (Alvarez *et al.,* 2015;
79 Hughes, Inouye, Johnson, Underwood, & Vellend, 2008; Schoener, 2011; Whitham *et al.*, 2006).

80 Additional layers of variation, including chromatin modifications, small RNAs, and other
81 non-coding variants, can mediate changes in genotypic expression and phenotype. However, this
82 type of variation is infrequently studied in natural settings (Alvarez *et al*. 2015; Kudoh 2016;
83 Nagano *et al*. 2012, 2019; Richards *et al.*, 2017). DNA and chromatin modifications, such as
84 DNA methylation, can also vary among individuals within populations (Banta & Richards, 2018;
85 Becker & Weigel, 2012; Richards *et al.*, 2017), and contribute to phenotypic variation by
86 modulating the expression of genes (Alvarez *et al*., 2015, 2018), the types of transcripts that
87 genes produce (Maor, Yearim, & Ast, 2015), the movement of mobile elements (Matzke &
88 Mosher, 2014), and the production of structural variants (Underwood *et al.*, 2018 ; Yelina *et al.*,
89 2015). At the same time, changes in genetic sequence or gene expression may cause variation in
90 patterns of DNA methylation, creating a bidirectional relationship that varies across the genome
91 (Meng *et al.*, 2016; Niederhuth & Schmitz, 2017; Secco *et al.*, 2015). Patterns of DNA
92 methylation have been correlated to habitat types, exposure to stress, and shifts in species range
93 (Foust *et al*., 2016; Liebl, Schrey, Andrew, Sheldon, & Griffith, 2015; Liebl, Schrey, Richards,
94 & Martin, 2013; Richards, Schrey, & Pigliucci, 2012; Verhoeven, Jansen, van Dijk, & Biere,
95 2010; Weyrich, *et al*., 2016; Xie *et al*., 2015). However, it is often unclear whether changes in
96 DNA methylation, and correlated changes in gene expression, are simply a downstream
97 consequence of changes in allele frequencies or if they may manifest through other mechanisms.

98 In 2010, the *Deepwater Horizon* (*DWH*) oil spill developed into the largest marine oil
99 spill in history (National Commission on the BP *Deepwater Horizon* oil spill, 2011), and became
100 an opportunity to test ecological and evolutionary hypotheses in a diversity of organisms
101 exposed to this recurrent anthropogenic stress (e.g. Alvarez *et al.* 2018; DeLeo *et al.* 2018;
102 Hazen *et al*. 2010; Kimes *et al*. 2013; Kimes, Callaghan, Suflita & Morris, 2014; Robertson,
103 Schrey, Shayter, Moss, & Richards, 2017; Rodriguez-R *et al.* 2015; Whitehead *et al*. 2012). A
104 mixture of crude oil and dispersants made landfall along 1,773 kilometers on the shorelines of
105 Louisiana, Mississippi, Alabama and Florida (Mendelssohn *et al.*, 2012; Michel *et al.,* 2013).
106 Nearly half of the affected habitat was salt marsh, which supplies valuable ecosystem functions
107 such as providing nurseries for birds and fish, and buffering storm and wave action (Day *et al.*,
108 2007; Mendelssohn *et al.*, 2012; Michel *et al.,* 2013). Gulf of Mexico salt marshes are dominated
109 by the hexaploid foundation plant species *Spartina alterniflora* (2n=6x=62; Marchant, 1968),
110 which is remarkably resilient to a variety of environmental stressors (Bedre, Mangu, Srivastava,
111 Sanchez, & Baisakh, 2016; Cavé-Radet, Salmon, Lima, Ainouche, & El Amrani, 2018; Pennings
112 & Bertness, 2001; Silliman *et al.*, 2012). Crude oil exposure from the *DWH* oil spill resulted in
113 reduced carbon fixation, reduced transpiration, and extensive above-ground dieback in *S.*
114 *alterniflora* populations (Lin & Mendelssohn, 2012; Silliman *et al.,* 2012), but oil-affected
115 populations showed partial to complete recovery within seven months of the spill (Lin *et al.,*
116 2016). However, the genomic and population level mechanisms that underlie this remarkable
117 recovery have been poorly characterized.
118 In previous studies, we found that in *S. alterniflora* exposed to the *DWH* oil spill,
119 pollution tolerance was correlated to changes in expression of a diverse set of genes, including
120 epigenetic regulators and chromatin modification genes, such as a homolog of SUVH5 (Alvarez
121 *et al.*, 2018). Although *S. alterniflora* populations were partially resilient to the *DWH* spill (Lin
122 & Mendelssohn, 2012), we found evidence of genetic differentiation between individuals from
123 oil-exposed areas and nearby uncontaminated areas (Robertson *et al.*, 2017). We expected that
124 DNA methylation patterns would be divergent between oil exposed and unexposed populations,
125 which might be induced by the environment or result from the genetic differences between
126 exposed and unexposed populations. However, while a few DNA methylation loci (measured via
127 methylation sensitive amplified fragment length polymorphism; MS-AFLP) were correlated with

128 oil exposure, we did not find genome-wide patterns in DNA methylation correlated with oil
129 exposure in *S. alterniflora* (Robertson *et al.*, 2017).
130 In this study, we used a recently developed reduced representation bisulfite sequencing
131 (RRBS) technique, epigenotyping by sequencing (epiGBS), to generate a more robust DNA
132 sequence and DNA methylation data set (van Gurp *et al.*, 2016). We expected that the increased
133 resolution, both in number and in detail of the markers, provided by this sequencing approach
134 would confirm our previously observed patterns of genetic differentiation, and allow us to
135 identify fine scale DNA methylation structure that was not apparent in our previous study. By
136 aligning our fragments to the *S. alterniflora* transcriptomes (Boutte *et al.*, 2016; Ferreira de
137 Carvalho *et al.,* 2013, 2017) and *Oryza sativa* genome (Kawahara *et al.*, 2013), we expected to
138 assess the relationship between DNA methylation and previously reported gene expression. We
139 predicted that we would find evidence that DNA methylation was correlated with changes in
140 gene expression since some fragments might overlap with the coding regions of genes
141 (Niederhuth & Bewick *et al.*, 2016). In addition, we examined the potential for response to
142 selection by crude oil exposure among genotypes collected from the field in a common garden
143 greenhouse experiment. We predicted that we would find variation in response to crude oil
144 among genotypes, which would indicate existing standing variation in wild populations of *S.*
145 *alterniflora* that could be acted upon by selection.
146
147 **Materials and Methods**
148
149 *Sample Collection*
150 We collected individuals from the leading edge of the marsh at three contaminated and
151 three neighboring uncontaminated sites near Grand Isle, Louisiana and Bay St. Louis,
152 Mississippi in August 2010, four months after the *DWH* oil spill as described in previous studies
153 (Table 1; Alvarez *et al.,* 2018; Robertson *et al.,* 2017). These sites were naturally variable in
154 conditions, but all sites supported monocultures of *S. alterniflora*. Contaminated sites were
155 identified by the visual presence of oil on the sediment and substantial above-ground dieback of
156 *S. alterniflora* on the leading edge of the marsh with *S. alterniflora* plants growing through the
157 dead wrack. Nearby uncontaminated sites did not have any visible signs of the presence of oil or
158 noticeable dieback of the above ground portions of *S. alterniflora*. Contamination status was

159 later confirmed via National Resource Damage Assessment databases (Robertson *et al.*, 2017).
160 To standardize age and minimize developmental bias in sampling, we collected the third fully
161 expanded leaf from each of eight individuals, spaced 10 meters apart at each of the six sites
162 (N=48). Leaf samples were immediately frozen in liquid nitrogen to prevent degradation, and
163 kept frozen during transport to the University of South Florida for processing and analysis.
164
165 *DNA extractions and library prep*
166 We isolated DNA from each field-collected sample (N=48) using the Qiagen DNeasy
167 plant mini kit according to the manufacturer's protocol. We prepared epiGBS libraries *sensu* van
168 Gurp *et al.* (2016). Briefly, isolated DNA was digested with the enzyme PstI, which is sensitive
169 to CHG methylation and biases resulting libraries toward coding regions (van Gurp *et al.,* 2016).
170 After digestion, adapters containing methylated cytosines and variable barcodes were ligated to
171 either end of the resulting fragments. We used the Zymo EZ Lightning methylation kit to
172 bisulfite treat and clean the DNA. Libraries were then amplified with the KAPA Uracil Hotstart
173 Ready Mix with the following PCR conditions: an initial denaturation step at 98ºC for 1 min
174 followed by 16 cycles of 98ºC for 15s, 60ºC for 30s, and 72ºC for 30s, with a final extension of
175 72ºC for 5 min. We used rapid run–mode paired-end sequencing on an Illumina HiSeq2500
176 sequencer using the HiSeq v4 reagents and the HiSeq Control software (v2.2.38), which
177 optimizes the sequencing of low-diversity libraries (van Gurp *et al.,* 2016).
178
179 *Data pre-processing and mapping to transcriptome*
180 We used the epiGBS pipeline (van Gurp *et al.,* 2016) to demultiplex samples, trim
181 adapter sequences, assemble the *de novo* reference sequence, and call single nucleotide
182 polymorphisms (SNPs) and DNA methylation polymorphisms (DMPs)
183 (https://github.com/thomasvangurp/epiGBS). Sequencing depth varied substantially between
184 samples, which we evaluated with a principal components analysis (PCA) on sampling depths
185 across loci. We assumed that an approximately even spread of the samples across PC1 and PC2
186 with no association of population or oil exposure, would indicate that sampling depth did not
187 bias our downstream analyses (Figure S1). SNPs (the resulting snps.vcf file) and DMPs
188 (methylation.bed) were filtered separately for each individual to include only loci that were
189 sequenced a minimum of ten times (10x depth of coverage), while loci below this coverage were

190 considered missing data. We first removed 10 individuals with high amounts of missing data
191 (>80%), leaving 38 samples across all 6 populations (Table 1). We then retained only loci that
192 were present in more than 50% of individuals, with no more than 70% missing from any one
193 individual (Figure S2). During the course of this filtering, missing data were imputed via a k-
194 nearest neighbors approach (impute, Hastie, Tibshirani, Narasimhan, & Chu, 2018). We also
195 performed genome-wide analyses (redundancy analyses, explained below) a second time with
196 stricter filtering parameters (no more than 50% missing data in any individual, and no more than
197 20% missing data at each locus, leaving 34 individuals) and obtained nearly identical P-values
198 and F-statistics, although percent variance explained was reduced (Supplementary File 1).
199 All fragments were mapped to the published *S. alterniflora* transcriptome (Boutte *et al.,*
200 2016) and the *O. sativa* genome (Michigan State University version 7, Kawahara *et al.*, 2013)
201 using BLAST (Altschul *et al.,* 1997). We used BLAST (Altschul *et al.,* 1997) and
202 RepeatExplorer (Novak, Neumann, Pech, Steinhaisl, & Macas*,* 2013) to compare our sequenced
203 fragments to the *S. alterniflora* transcriptome (Boutte *et al.*, 2016; Ferreira de Carvalho *et al.,*
204 2013, 2017) and known repeat elements, respectively.
205
206 *Population genetics*
207 All statistical analyses were performed in R v 3.5.3 (R Core Team, 2017). The epiGBS
208 technique, and the sequencing design that we chose, did not provide sufficient sequencing depth
209 to estimate hexaploid genotype likelihoods with confidence, particularly considering the lack of
210 a high-quality reference genome (Boutte *et al.,* 2016; Dufresne *et al.,* 2014). We therefore used
211 the frequency of the most common allele within an individual at each polymorphic locus as a
212 substitute for genotype at each locus. Although this method ignores the various types of partial
213 heterozygosity that are possible in hexaploid *S. alterniflora*, methods do not currently exist for
214 accurate estimation of heterozygosity in polyploids, and the majority of standard population
215 genetic inference assumes diploidy. We assumed that the frequency of the most common allele
216 was likely to underestimate diversity and therefore underestimate divergence between
217 populations, making our tests of differentiation conservative (Meirmans, Liu, & van Tienderen,
218 2018).
219 We obtained pairwise F_{ST} values between populations to test for significant
220 differentiation (StAMPP, Pembleton, Cogan, & Forster*,* 2013). We also used distance-based

221 redundancy analysis (RDA function in the Vegan package v. 2.5-2; Oksanen *et al.* 2017) to
222 minimize false positives (Meirmans, 2015) in assessing isolation by distance using the formula
223 (genetic distance ~ latitude * longitude). We visualized data using principal components analysis
224 (PCA; Figure 1A).
225 To quantify the relationship between genome-wide variation and environmental
226 conditions, we used partial constrained redundancy analysis (RDA, implemented with the RDA
227 function in the Vegan package v. 2.5-2; Oksanen *et al.* 2017). RDA is a multivariate ordination
228 technique that allowed us to assess the joint influence of all SNPs simultaneously, while
229 effectively controlling for both population structure and false discovery (Forester, Lasky,
230 Wagner, & Urban, 2018). The resulting "locus scores" correspond to the loadings of each SNP
231 on to the constrained axis, which represents the variation that can be explained by the variable of
232 interest (in this case, crude oil exposure). We attempted to control for variation among sites with
233 a replicated sampling strategy, but rather than using a single term for "population", we
234 conditioned our ordination on variables identified by latent factor mixed models analysis using
235 the LFMM package (Caye, Jumentier, Lepeule, & François, 2019), which provides a method to
236 account for residual variation due to unmeasured differences among populations, including
237 environmental variation, life history variation, and geographical separation (Leek *et al.*, 2017).
238 We used RDA to fit our final model with the formula (SNP matrix ~ oil exposure +
239 Condition(latent factors)). We used a permutational test (999 permutations; Oksanen *et al.* 2017)
240 to assess the likelihood that oil-exposed and unexposed populations differed by chance, and
241 visualized results using principal components analysis. We identified individual SNPs that were
242 significantly correlated with oil contamination using the three standard deviation outlier method
243 described by Forester *et al*. (2018). Finally, we tested for differences in genetic variation using
244 the PERMDISP2 procedure (Vegan; Oksanen *et al*., 2017), under the assumption that a
245 significant reduction in genetic variation in oiled populations may be evidence of a bottleneck.
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 multiple testing correction after
250 locus-by-locus modeling. We tabulated methylation frequency at each locus (methylated

251 cytosines/(methylated+unmethylated cytosines)), and visualized differences between samples
252 with PCA (Figure 2A).
253 To identify signatures of DNA methylation variation that were correlated with oil
254 exposure while controlling for genetic structure, we estimated latent variables with LFMM (Caye
255 *et al.,* 2019) as above. In addition to the advantages described above, latent factor analysis (or the
256 related surrogate variable analysis) provides a control for cell type heterogeneity in epigenomic
257 studies (Akulenko, Merl, & Helms, 2016; Caye *et al.,* 2019; McGregor *et al.,* 2016). We then
258 modeled the impact of oil exposure to genome-wide patterns of DNA methylation while
259 controlling for latent variation as well as population structure via RDA (Vegan v. 2.5-2; Oksanen
260 *et al.,* 2017) with the formula (methylation distance ~ oil exposure + Condition(latent factors) +
261 the first 5 principal components).
262 To identify differentially methylated positions (DMPs) between contaminated and
263 uncontaminated samples, we used binomial linear mixed modeling (MACAU; Lea, Tung, &
264 Zhou, 2015), using the genetic relatedness matrix and latent factors as covariates to control for
265 population structure. We corrected locus-specific P-values for multiple testing (qvalue v 2.14.1;
266 Storey, Bass, Dabney, & Robinson, 2015), and tested for overrepresentation of cytosine contexts
267 (CG, CHG, CHH) using binomial tests, implemented in R (R Core Team, 2017). Our epiGBS
268 fragments rarely exceeded 200bp, and we were therefore unable to identify differentially
269 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 associated with response to the *DWH* in 3,334 out of 15,950 genes that
274 were assessed in *S. alterniflora* (Alvarez *et al*., 2018). In order to make the epiGBS data
275 comparable to our pooled microarray design, we concatenated SNPs and methylation
276 polymorphisms from individuals into *in silico* sample pools by averaging values at individual
277 loci across the same three individuals within pools that were used in the gene expression
278 analysis. We then calculated genetic, expression, and methylation distances between sample
279 pools and used Mantel and partial Mantel tests to assess the relationship between all three data
280 types and between methylation and expression, controlling for the effect of genetic distance
281 (Vegan, Oksanen *et al.* 2017).

282 We also obtained the probability of whether significantly associated SNPs and
283 methylation positions were likely to be overlapping differentially expressed genes (DEGs) by
284 chance using a bootstrap method. We drew a number of random SNPs or methylated positions,
285 with replacement, equal to the number of observed significantly associated SNPs or DMPs, and
286 counted the number of loci that overlapped with DEGs in our previous study. This process was
287 repeated 9999 times each for genetic and methylation data. We derived P-values by counting the
288 number of times a value at least as large as the observed value appeared in the bootstrap
289 resamples and dividing by the number of bootstrap replicates.
290
291 *Greenhouse oil response experiment*
292 We assessed the possibility that native *S. alterniflora* populations harbored genetic
293 variation for the response to crude oil via a controlled greenhouse experiment. We collected 10
294 *S. alterniflora* individuals that had been collected from two oil-naïve sites ("Cabretta" and
295 "Lighthouse") in the Sapelo Island National Estuarine Research Reserve in Georgia, USA, in
296 May 2010. We collected plants that were spaced ten meters apart, maximizing the chance that
297 individuals were of different genotypes (Foust *et al.,* 2016; Richards, Hamrick, Donovan, &
298 Mauricio, 2004). We grew these individuals in pots under greenhouse conditions for at least
299 three years before beginning our experiments and propagated multiple replicates of each
300 genotype by rhizome cutting. Individual ramets were separated and potted in 4 inch pots in a 50-
301 50 mixture of peat and sand (Cypress Creek, Tampa, USA; Alvarez, 2016).
302 We distributed three potted replicates of each of the 10 genotypes in each of two plastic
303 containment chambers, for a total of 60 biological samples. One chamber received only
304 uncontaminated fresh water, while the oil treatment chamber received 2.5% oil (sweet Louisiana
305 crude) in 62 liters of water, which we previously determined would induce strong phenotypic
306 response (Alvarez, 2016). Tides were simulated once per day by filling containment chambers
307 with the water or water-oil mixture and allowing the fluid to drain into a catchment. We
308 estimated biomass by tallying the number of living leaves and the number of living ramets when
309 the experiment began, and again seven days after crude oil was added.
310
311 *Statistical analysis of greenhouse experiment*

312 We used generalized linear models (R Core Team 2017) with a Poisson error distribution
313 to analyze the above-ground biomass at the end of the experiment (measured as the number of
314 leaves and the number of ramets). Because *S. alterniflora* reproduces clonally, we assumed that
315 biomass would represent a reasonable proxy of fitness in our species (Younginger, Sirová,
316 Cruzan, & Ballhorn, 2017). We also included a covariate for the size of each plant at the start of
317 the experiment, represented by the number of leaves and the number of ramets at the start of the
318 experiment. Each model was written as ($\text{Phenotype}_{\text{Final}}$ ~ Treatment * Genotype +
319 $\text{Phenotype}_{\text{Initial}}$), where asterisks represent main effects and all interactions. We did not explicitly
320 test for differences between sites since admixture is high between sites on Sapelo Island and we
321 found no evidence of genetic differentiation (Foust *et al*., 2016). We assessed significance for
322 main effects and interactions using type III tests. However, to identify individual genotypes
323 responding more strongly than others, we conducted post-hoc pairwise comparisons, correcting
324 for multiple testing using Holm's correction for multiple testing (emmeans; Lenth, 2018).
325
326 **Results**
327
328 *epiGBS yields informative genetic and methylation loci*
329 The libraries for 48 individuals (Table 1) generated 6,809,826 total raw sequencing reads,
330 of which 3,833,653 (56.3%) could be matched to their original mate strand. *De novo* assembly
331 using the epiGBS pipeline resulted in 36,131 contiguous fragments ranging from 19-202 bp, an
332 average length of 123.92 bp, and a total length of 5,441,437 bp. The size of the *S. alterniflora*
333 genome is estimated to be 2C values = 6x = 4.2 GB (Fortune *et al.*, 2008), and current genomic
334 analyses indicate that repetitive sequences (including transposable elements and tandem repeats)
335 represent about 45% of the total analyzed genomic data set in *S. alterniflora* (Giraud *et al., in*
336 *prep*). Therefore, we estimate that our epiGBS approach assayed approximately 0.6% of the non-
337 repetitive genome. However, fragments that were >90% similar were merged, and polyploid
338 homeologs may have been concatenated. With BLAST, we found 10,103 fragments mapped to
339 2,718 transcripts in the *S. alterniflora* transcriptome. We found that 1,571 transcripts (57.8%)
340 contained multiple epiGBS fragments that align to the same place, and 296 (10.9%) contained
341 multiple epiGBS fragments that mapped to different places within the same gene. We suspect
342 that multiple epiGBS fragments map to the same location because some epiGBS fragments

343 represent different homeologs of the same region, which mapped to the same location. Only 1%
344 of reads map to repetitive elements, confirming that *Pst1*-fragmented libraries were biased away
345 from highly methylated, repetitive regions (van Gurp *et al.,* 2016). The bisulfite non-conversion
346 rate was calculated to be 0.36% of cytosines per position, and was estimated from lambda phage
347 spike-in (van Gurp *et al.,* 2016). Although we found substantial variation in average sequencing
348 depth among samples, we found no obvious non-random bias in sampling depth across samples
349 (Figure S1). However, during filtering, we removed ten samples due to stochastic under-
350 sequencing, leaving 38 samples for population analyses (Table 1, Figure S2).
351
352 *Genetic differentiation*
353 Our initial sequencing run yielded 171,205 SNPs across all individuals. After filtering to
354 common loci, removing invariant sites, and imputing missing data (Figure S2), we obtained
355 63,796 SNP loci. Of these, 5,753 SNPs occurred in transcripts. As in our AFLP study, we found
356 significant genetic differentiation that was correlated to oil exposure: oil exposure explained
357 23.4% of the variance in DNA sequence ($P<0.001$, Figure 1A, B, Table 2), providing evidence
358 that selection may have acted on these populations. Pairwise F_{ST} calculations showed that all
359 sites were significantly different from each other (Table 3), with no evidence of isolation by
360 distance ($P>0.05$ for latitude, longitude, and interaction). We found 1,631 SNPs that were
361 significantly associated with oil exposure (defined by a locus score >3 standard deviation units
362 away from the mean locus score; Forester *et al*., 2018; Figure 1C), including 169 that overlapped
363 with the *S. alterniflora* transcriptome. Of these loci, 41 were annotated using information from
364 *O. sativa,* and contained a number of putative regulators of gene expression. Among significant
365 loci, 1,324 differed in major allele frequency between exposed and unexposed populations by
366 greater than 5%, and 334 by greater than 20% (Figure 1C). Significantly differentiated loci
367 appeared no less likely to increase or decrease in major allele frequency based on exposure (809
368 increasing vs 822 decreasing in frequency). We tested for homogeneity of group dispersion, and
369 found no evidence of change in variance in oil-exposed populations ($P=0.512$).
370
371 *DNA methylation differentiation*
372 Our bisulfite sequencing yielded 1,402,083 cytosines that were polymorphic for
373 methylation across our samples before filtering. After filtering our data to common loci as

374 described above, we analyzed 92,999 polymorphic methylated loci, 25,381 of which occurred in
375 the CG context, 24,298 in the CHG context, and 43,030 in the CHH context (Figure 2C). An
376 additional 290 had variable context because they co-occurred with a SNP. These proportions of
377 polymorphic methylation loci did not change significantly due to oil exposure. Methylation calls
378 were collapsed for symmetric CG and CHG loci across "watson" and "crick" strands so that
379 methylation on either one or both strands was considered as a single locus. Although DNA
380 methylation was strongly correlated with oil exposure (Table 2, $P<0.001$) when controlling only
381 for latent factors, this differentiation was not significant after controlling for genetic population
382 structure with principal components of genetic data (Table 2, $P>0.1$). In the latter model, oil
383 explained 10% of the variation in methylation.
384 We found 240 DMPs that differed significantly between exposure types ($Q<0.05$, Figure
385 2C; Table S1). The number of observed DMPs in the CG context (125 loci) was significantly
386 overrepresented relative to their occurrence in our data ($P<0.001$). We also found DMPs in CHG
387 (57 loci), and CHH (58 loci) context, which was underrepresented among DMPs relative to their
388 prevalence in all contigs ($P<0.001$). Among the significant loci, most had negligible differences
389 in the magnitude of methylation frequency changes (average 1.4% change between exposed and
390 unexposed populations). Only 29 experienced a change in magnitude of methylation greater than
391 5%, and only 7 loci showed a change of greater than 20%. Additionally, 19 DMPs were located
392 within a fragment that mapped to the *S. alterniflora* transcriptome, and 49 DMPs occurred in the
393 same fragment as a significantly differentiated SNP. However, only 4 of those SNPs altered the
394 trinucleotide context of DNA methylation.
395
396 *Correlations with gene expression*
397 We found no significant relationship between genetic distance and gene expression
398 distance (Mantel's $R= 0.050$, $P=0.32$), between patterns of methylation variation and genome
399 wide gene expression (Mantel's $R=0.051$, $P=0.29$), or between methylation and genome wide
400 expression when controlling for genetic variation (Mantel's $R=0.014$, $P= 0.41$). Only 14 SNPs
401 that were significantly associated with oil exposure overlapped with DEGs correlated with
402 exposure to the *DWH* oil spill (Alvarez *et al.,* 2018). However, our bootstrap test showed that
403 this overlap could occur by chance ($P>0.79$). Therefore, our data suggests that if these SNPs are
404 under selection, they are not necessarily regulating differential expression resulting from coding

405 changes in those genes. In addition, although 19 DMPs overlapped coding regions, only 3 of the
406 DMPs corresponded to a DEG (Table S1), and our bootstrap test suggests that this was also
407 likely to occur by chance (P>0.5). However, our data is limited to address the association
408 between DNA methylation and differential expression of specific genes.
409
410 *Genotypes in common garden differ in their response to crude oil*
411 We found a significant effect of oil exposure on both the number of leaves (F= 13.09, P <
412 0.001) and the number of ramets (F = 28.75, P < 0.001) at the end of the controlled greenhouse
413 experiment. Type III tests showed significant genotype-by-treatment interactions for the number
414 of leaves, but not ramets, at the end of the experiment, suggesting that individual genotypes vary
415 in their response to crude oil exposure. Post-hoc comparisons identified two genotypes (C and G;
416 FDR<0.05, Figure 3**;** Table S2) that lost a significantly greater number of leaves over the course
417 of the experiment relative to other genotypes, further suggesting the presence of standing
418 variation among individuals for the response to crude oil exposure.
419
420 **Discussion**
421 *Spartina alterniflora* displays high levels of genetic and DNA methylation variation
422 across environmental conditions in its native range (Foust *et al.,* 2016; Hughes & Lotterhos,
423 2014; Richards *et al.*, 2004; Robertson *et al.*, 2017), potentially providing substrate for both
424 genetic and epigenetic response to pollution. We previously found that genetic structure and
425 expression of 3,334 genes were correlated to exposure to the *DWH* oil spill, but genome-wide
426 methylation variation was not (Alvarez *et al.*, 2018; Robertson *et al.,* 2017). Higher resolution
427 epiGBS suggests that both genetic sequence and DNA methylation are correlated with crude oil
428 exposure in *S. alterniflora*, but that differentiation in DNA methylation is primarily explained by
429 differences in allele frequencies. Additionally, our greenhouse experiment shows phenotypic
430 plasticity and genotypic variation in crude oil response, as measured by differential reduction in
431 biomass between exposed and unexposed samples. These findings are consistent with genotype-
432 specific mortality, and suggest that the *DWH* oil spill may have been a selective event in *S.*
433 *alterniflora* populations.
434
435 *Genetic and epigenetic response to the DWH*

436 We found significant genetic differentiation between oil-exposed and unexposed sites,
437 which may reflect either stochastic mortality in oil-exposed areas from a severe bottleneck, or a
438 signature of selection for oil tolerance in affected populations. *Spartina alterniflora* displays high
439 phenotypic plasticity, and populations have persisted after exposure to the *DWH* oil spill, even
440 after extensive aboveground dieback (Lin & Mendelssohn, 2012; Lin *et al.*, 2016; Silliman *et al.*,
441 2012). However, our studies and previous accounts of initial losses in live aboveground and
442 belowground biomass (Lin *et al*., 2016) suggest that some *S. alterniflora* genotypes were more
443 susceptible than others to crude oil stress, and either had not regrown at the time of sampling or
444 experienced mortality as a result of oil exposure. Although we found no evidence for a reduction
445 in genetic variation, which may have further indicated selection for tolerant genotypes, the high
446 ploidy of *S. alterniflora* makes accurate quantification of total genetic variation challenging.
447 Further investigations are required to confirm the magnitude of selection, whether mortality
448 varied by genotype, and if there was a reduction in genetic variation among oil-exposed
449 populations.
450 DNA methylation differences may reflect either the downstream effects of genetic
451 variants, an induced response to environment, or both (Meng *et al.*, 2016). For example, in
452 another study of *S. alterniflora* populations, patterns of DNA methylation were more strongly
453 correlated than genetic structure with microhabitat, and correlation of DNA methylation to
454 environment was independent of population structure (Foust *et al.,* 2016). In this study, we found
455 a multi-locus signature of methylation differentiation (17% of the variation explained by oil
456 exposure) between oil-affected and unaffected sites before controlling for population structure.
457 However, we found no association between methylation and crude oil contamination after
458 controlling for genetic variation and latent effects, suggesting DNA methylation is controlled by
459 genetic variation.
460 The observed variation in DNA methylation may be controlled by genetic variation via
461 either a change in the nucleotide context, the presence or absence of particular alleles in *cis,* or
462 variation in upstream regulatory elements. Allelic variation that changes trinucleotide context
463 can alter or eliminate the ability of a methyltransferase to deposit methylation at that site.
464 However, in our data, we did not find an enrichment of SNPs that affected trinucleotide context
465 of DMPs. Concurrently, we did not detect an enrichment of oil-associated SNPs in DEGs, which
466 we would expect if changes in the coding regions of those genes explain the observed gene

467 expression variation in oil-exposed individuals. However, our ability to assess the relationships
468 between SNPs, SMPs and DEGs was limited by the distribution of our RRBS fragments. Further,
469 changes in allele frequencies, due to either selection or drift, may have generated divergence in
470 the regulatory machinery maintaining DNA methylation and gene expression profiles among
471 exposed and unexposed populations.
472 Although we cannot disentangle whether differential expression causes alternative
473 methylation patterns or vice versa, we previously identified a DEG that was homologous to the
474 histone methyltransferase SUVH5, which may modulate fitness effects during oil exposure
475 (Alvarez *et al.,* 2018). Histone methylation is linked to DNA methylation through the regulation
476 of CHROMOMETHYLASE3 (CMT3) activity (Stroud, Greenberg, Feng, Bernatavichute, &
477 Jacobsen, 2013). Given our previous results and those from the present study, we hypothesized
478 that the differential expression of SUVH5 in response to crude oil exposure would result in
479 differences in DNA methylation. These differences, in turn, may be maintained via genetic
480 variation between exposed and unexposed populations either in the SUVH5 homolog itself, or
481 more broadly within the CMT3-mediated pathway. However, targeted resequencing and further
482 functional validation in the populations of interest will be required to confirm this hypothesis.
483
484 *Reduced representation sequencing compared to AFLP*
485 As the field of ecological genomics matures, there is a pressing need to develop robust
486 assays and statistically sound measures of regulatory variation. Reduced representation
487 methylation sequencing techniques are attractive for ecological epigenomics because they can be
488 used to infer genome wide patterns of both genetic and methylation variation without a high-
489 quality reference genome (Paun, Verhoeven, & Richards, 2019; Richards *et al.*, 2017; Robertson
490 & Richards, 2015; van Moorsel *et al.* 2019). However, they still have serious limitations
491 particularly for species that do not yet have a fully sequenced reference genome (Paun *et al.*,
492 2019). Furthermore, it is important to note that the limited number of loci surveyed may have led
493 to a biased subsampling of the genome. In turn, this can lead to a poor estimation of the "neutral"
494 level of divergence in the genome, and therefore a biased interpretation of divergence between
495 these populations (Lowry *et al*., 2017).
496 When comparing epiGBS to MS-AFLP, we expected that the substantial increase in
497 markers (92,999 compared to 39 polymorphic methylation loci, respectively) would lend greater

498 resolution to detect patterns of DNA methylation variation. Our epiGBS survey detected
499 significant differentiation in both genetic variation and DNA methylation that was correlated to
500 oil exposure, suggesting that epiGBS provides increased resolution over MS-AFLP to detect
501 genome-wide methylation differences. However, despite the much larger data set generated by
502 epiGBS, we only found 240 differentially methylated positions. Although it would be valuable to
503 identify associations between gene expression and nearby DNA methylation variation, the
504 minimal overlap between our RRBS fragments and DEGs hindered our ability to associate
505 methylation and gene expression variation. This is due to the small fraction of the genome that is
506 assayed, substantial variation in methylation, and that we were unable to identify fragments that
507 overlapped promoter regions without a reference genome.
508 Future RRBS studies will benefit from optimizing protocols that enrich for specific
509 portions of the genome (*e.g.* Heer & Ullrich *et al.,* 2018), but generating a draft reference
510 genome will be imperative to allow for better exploitation of RRBS data and ascertain gene
511 function (Paun *et al.*, 2019). While sequencing-based techniques provide the potential to identify
512 functional genomic regions, correct annotations rely on genomic resources in a relevant
513 reference. In polyploid species like *S. alterniflora*, the number of duplicated genes and the
514 potential for neofunctionalization among them creates additional uncertainty for correctly
515 assigning annotations (Primmer, Papkostas, Leder, Davis & Ragan, 2013). *Spartina alterniflora*
516 has various levels of duplicated gene retention, small RNA variation (including miRNAs and
517 SiRNAs) and homeologous expression (Ainouche, Baumel, Salmon, & Yannic 2003; Boutte *et*
518 *al.*, 2016; Cavé-Radet, Giraud, Lima, El Amrani, Ainouche, & Salmon, 2019; Ferreira de
519 Carvalho, 2013, 2017; Fortune *et al.*, 2007), which may result in more opportunities for gene
520 diversification and subfunctionalization (Chen *et al.*, 2015; Salmon & Ainouche, 2015; Shimizu-
521 Inatsugi *et al.,* 2017). Therefore, while studies with RRBS techniques in non-model plants offer
522 increased power to detect broad, genome-wide patterns of variation that may be correlated to
523 ecology, they are still limited for the detection of specific gene function. Improving genomics
524 resources in a variety of organisms is an essential next step for understanding the molecular level
525 basis of ecological interactions.
526
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Tables and Figures

Table 1 Sampling information across all sites after filtering. Site information includes location and oil status at each site (exposure).

Site Location	Site Code	Exposure	No. of individuals
Grand Isle, LA	GIN1	None	8
Grand Isle, LA	GIN2	None	6
Barataria Bay, LA	GIO1	Heavily Oiled	3
Barataria Bay, LA	GIO2	Heavily Oiled	7
Bay St. Louis, MS	MSN	None	6
Bay St. Louis, MS	MSO	Heavily Oiled	8

Table 2 Association between oil exposure, genetic distance, and methylation distance across tests. Test statistics and significance determined through RDA. *** indicates significance at $P \leq 0.001$.

	df	F		Variance Explained
Genetic	1	2.183	***	0.234
Methylation				
Without control for genetic var.	1	1.96	***	0.167
With control for genetic var.	1	1.199		0.099

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

	GIN1	GIN2	MSN	GIO1	GIO2
GIN2	**0.0855**				
MSN	**0.1045**	**0.1219**			
GIO1	**0.0707**	**0.0909**	**0.1088**		
GIO2	**0.0885**	**0.1091**	**0.1251**	**0.0914**	
MSO	**0.1108**	**0.1343**	**0.1507**	**0.1106**	**0.1330**

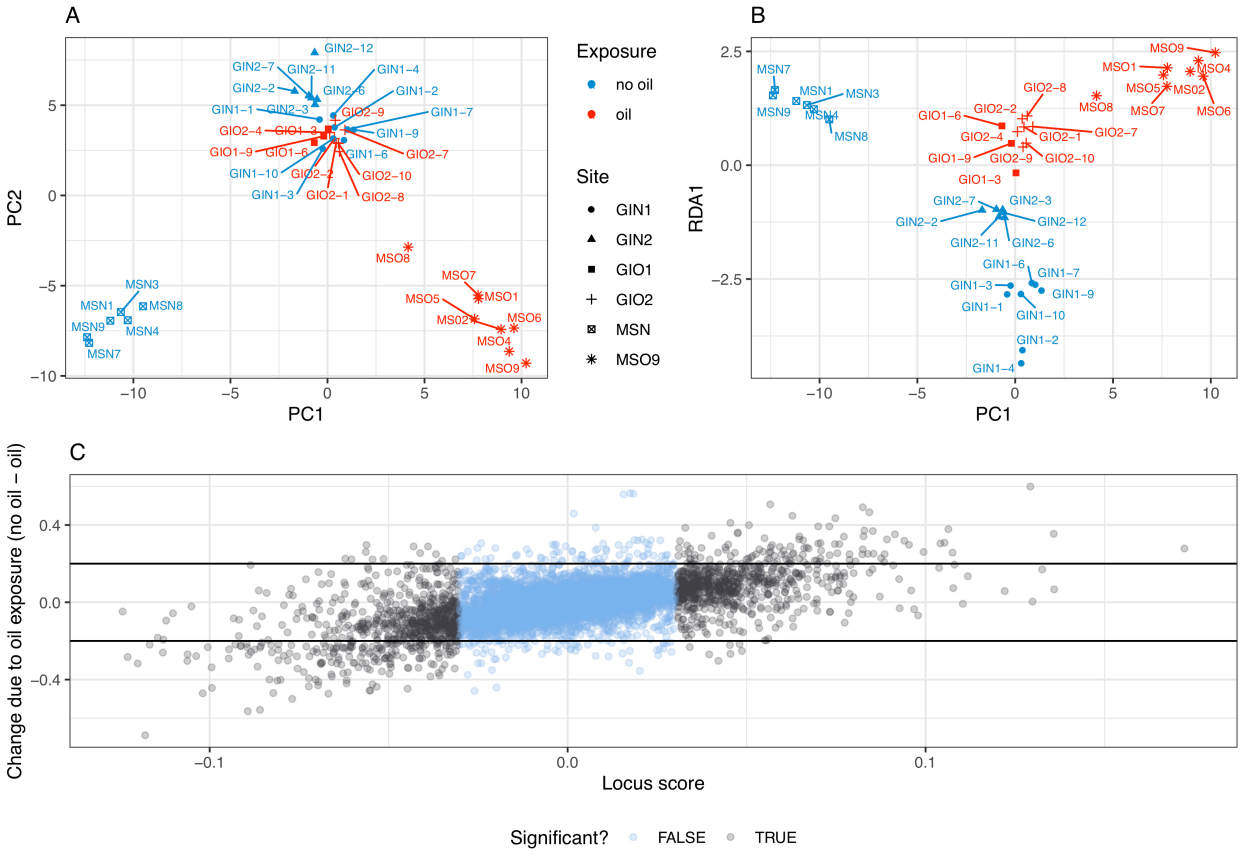

Figure 1 A) Visualization of principal components 1 and 2 of allele frequency data (SNP) data. B) Visualization of principal component 1 and RDA1, which represents the line of maximal separation between samples based on allele frequency data. C) The locus score, representing loadings of each SNP to the constrained axis, plotted against the average change in allele frequency between unexposed and oil exposed populations. Significantly differentiated SNPs are shown in black.

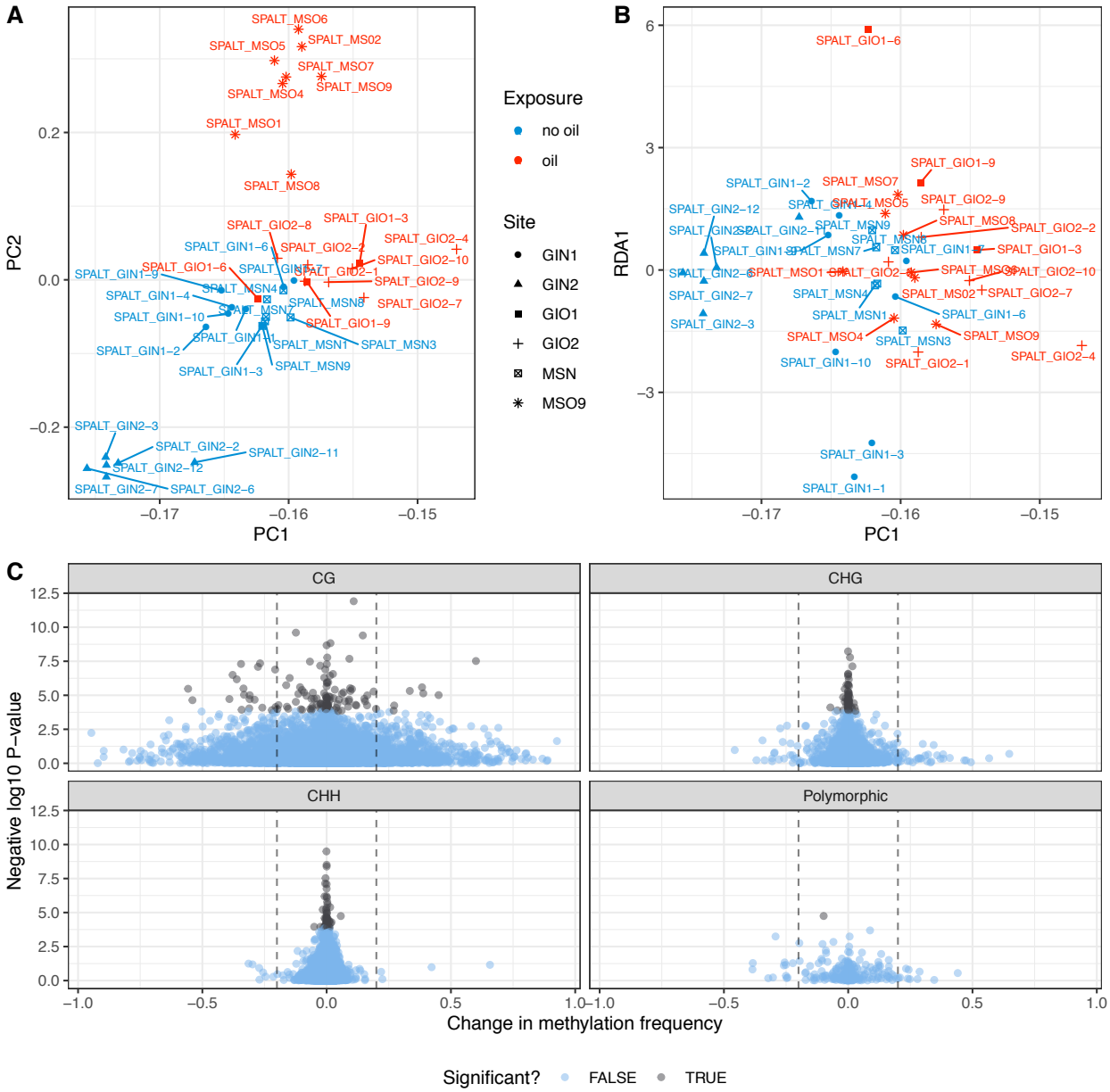

Figure 2 A) Visualization of principal components 1 and 2 of methylation frequency data. B) Visualization of principal component 1 and RDA1, which represents the line of maximal separation between samples based on methylation frequency data, after controlling for genetic variation and latent factors. C) All methylation polymorphisms, with differentially methylated positions (DMPs) shown in black. Negative log10 P-values are plotted on the Y axis while the average change in methylation frequency between unexposed and oil exposed populations is shown on the X axis. Dotted lines represent a change of at least 20%, either increased or decreased, due to oil exposure.

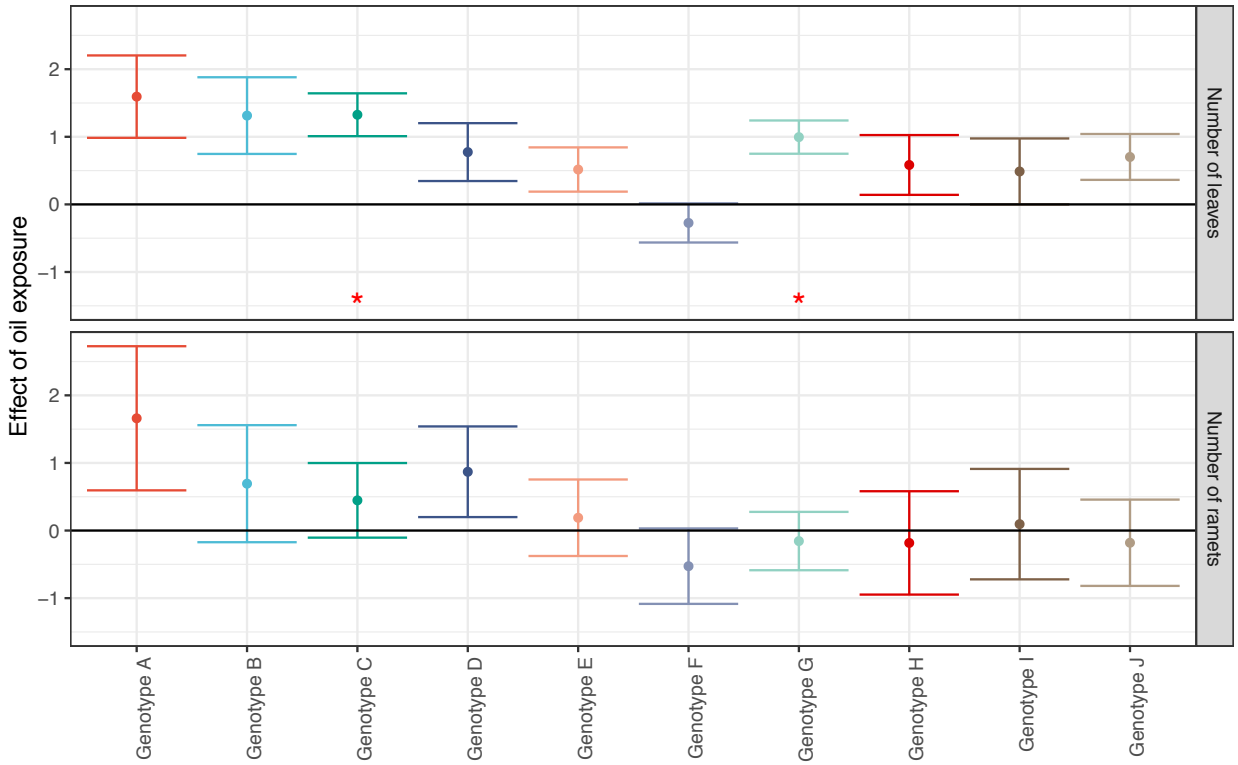

Figure 3 Variation in effect size estimates of the effect of crude oil exposure in individual genotypes. Estimates were based on estimated marginal means in our greenhouse experiment. Asterisks represent significance in post-hoc comparisons.

929 **Author Contributions**

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

935

936 **Data accessibility**

937 Raw data files are available on Dryad at XXX. Supplementary tables and figures can be
938 found in the electronic supplementary material, and is available on
939 github.com/alvarezmf/DWH_epigbs along with processed data and R scripts.

940

941 **Supporting Information**

942 Table S1 SNPs and methylation loci significantly associated with oil exposure.
943 Table S2 Post-hoc tests for the effect of crude oil exposure in individual genotypes. Estimates
944 were based on estimated marginal means in our greenhouse experiment.
945 Figure S1 A) Principal components analysis on sampling depth per SNP allele. B) Percent
946 variance explained by each principal component.
947 Figure S2 Upper half: percentage of present and imputed data per sample after filtering for A)
948 SNP and B) methylation loci. Lower half: percentage of present and imputed data per locus after
949 filtering for C) SNP and D) methylation loci. In C and D, only the first 5,000 loci are shown for
950 clarity. Dotted lines represent missing data cut-off values for removal (80% missing for
951 individuals in A & B, 50% for loci in C & D).