

DNA methylation variation in gametes and larvae of the Pacific oyster, *Crassostrea gigas*

Claire E. Olson

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Steven Roberts, Chair

Graham Young

Lorenz Hauser

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Claire E. Olson

University of Washington

**Abstract**

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Claire E. Olson

Chair of Supervisory Committee:  
Assistant Professor Dr. Steven B. Roberts  
School of Aquatic and Fishery Sciences

Epigenetics describes DNA modifications that change gene expression without altering the underlying nucleotide sequence. Epigenetic mechanisms such as DNA methylation can change genome function under external influences. The focus of this project is examining one epigenetic modification, DNA methylation, in oysters. DNA methylation has been well studied in vertebrates, but remains understudied in invertebrates. Furthermore, the amounts and functions of DNA methylation in organisms are extremely diverse and variable across taxa. This thesis determines patterns of DNA methylation in *C. gigas* to elucidate the functional role of DNA methylation. The first chapter examines the genome-wide DNA methylation profile in *C. gigas* male gamete cells using whole-genome bisulfite sequencing. RNA-Seq analysis was also performed on the same tissue to provide insight into the mechanisms by which DNA methylation impacts transcriptional processes. The work presented in Chapter 2 examines methylation patterns of *C. gigas* during early oyster developmental stages (spermatozoa and larvae). Together these data were used to test the predictions that DNA methylation is involved in gene regulatory activity and is heritable. This work also describes individual variation, parental transmission and developmental patterns of DNA methylation in oysters. Our results indicate a positive relationship between DNA methylation and gene expression, and that DNA methylation patterns are inherited in oysters.

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## **Chapter I: Genome-wide profiling of DNA methylation and gene expression in *Crassostrea gigas* male gametes**

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### ***Abstract***

DNA methylation patterns and functions are variable across invertebrate taxa. In order to provide a better understanding of DNA methylation in the Pacific oyster (*Crassostrea gigas*), we characterized the genome-wide DNA methylation profile in male gamete cells using whole-genome bisulfite sequencing. RNA-Seq analysis was performed to examine the relationship between DNA methylation and transcript expression. Methylation status of over 7.6 million CpG dinucleotides was described with a majority of methylated regions occurring among intragenic regions. Overall, 15% of the CpG dinucleotides were determined to be methylated and the mitochondrial genome lacked DNA methylation. Integrative analysis of DNA methylation and RNA-Seq data revealed a positive association between methylation status, both in gene bodies and putative promoter regions, and expression. This study provides a comprehensive characterization of the distribution of DNA methylation in the oyster male gamete tissue and suggests that DNA methylation is involved in gene regulatory activity.

### ***Introduction***

DNA methylation is an important epigenetic process that varies in genomic distribution and biological function across taxa. DNA methylation involves the addition of a methyl group to a cytosine pyrimidine ring and most often occurs as part of C-G

nucleotide pairs, frequently referred to as CpG dinucleotides. Mammals exhibit a pattern commonly referred to as global methylation, in which 70–80% of CpG dinucleotides are methylated (Bird, 1980). In contrast, invertebrates display relatively low levels of DNA methylation, from almost no methylation in *Drosophila melanogaster* (Gowher et al., 2000) to intermediate levels in the sea urchin *Echinus esculentus* (Bird et al., 1979). In mammals, a primary function of DNA methylation is to suppress gene expression through increased promoter DNA methylation (Bell and Felsenfeld, 2000). However, the function of DNA methylation in invertebrates is variable and likely differs among invertebrate taxa. The roles of methylation include regulation of transcriptional activity (Suzuki and Bird, 2008), alternatively exon splicing (Lyko et al., 2010), and developmental activity (Riviere et al., 2013).

While in general there is a limited amount of comprehensive information regarding DNA methylation in non-mammalian taxa, some recent studies have focused on DNA methylation in the Pacific oyster. The Pacific oyster is an excellent model for studying epigenetic modifications because its life history characteristics make it an important aquaculture species (Glude and Chew, 1982) and genomic resources for this species have recently become available (Zhang et al., 2012). Gavery and Roberts (2010) first reported the presence of DNA methylation in the Pacific oyster. In the same study, *in silico* analysis revealed a significant correlation between gene function and methylation level (Gavery and Roberts, 2010). The relationship between gene methylation and gene function was experimentally corroborated with high-throughput sequencing and it has been proposed that limited methylation in select genes may contribute to increased phenotypic plasticity in highly fluctuating environments (Roberts and Gavery, 2012).

More recently, methylation enrichment and bisulfite sequencing were used to describe high-resolution DNA methylation patterns in pooled oyster gill tissue (Gavery and Roberts, 2013). A characterization of DNA methylation during oyster larval development has also been performed, revealing that DNA methylation varies through early development and treatment with 5-Aza-cytidine, a DNA methyltransferase (DNMT) inhibitor, leads to developmental alterations (Riviere et al., 2013). In the same study, researchers found an inverse correlation between methylation proximal to the transcription start site and expression of *Hox* genes (Riviere et al., 2013). In addition to studies that investigate putative function of DNA methylation, other research has begun to evaluate relationships between epigenetic and genetic variations in *C. gigas* mass selection procedures (Jiang et al., 2013).

While a better understanding of DNA methylation is emerging for this species, there are still several questions that remain. Importantly we still do not fully understand the relationship between DNA methylation and gene expression, nor DNA methylation patterns in a single cell type. Examining a single cell type is important as methylation levels and patterns may differ between multiple cell types and life history stages, and our research attempted to limit this potential variability. Spermatozoa are an ideal resource for studying a single cell type and also provide the secondary benefit of understanding more about oyster spermatogenesis. The oyster male gonad consists of numerous gonadal tubules that grow during tissue development (Franco et al., 2008) and evolve according to four successive reproductive stages annually (Berthelin et al., 2000). These gonadal stages include undifferentiated (stage 0), mitosis of spermatogonia and differentiation of germ cells (stage 1), visible spermatogenesis (stage 2), and mature gametes (stage 3)

(Franco et al., 2008). This is the first time DNA methylation has been characterized in Pacific oyster gametes, however spermatozoa methylation has been previously examined in other marine invertebrates. For example, spermatozoa DNA methylation has been described in both the marine annelid worm *Chaetopterus variopedatus* (del Gaudio et al., 1997) and *Ciona intestinalis* (Suzuki et al., 2013).

This research represents the first high-resolution characterization of DNA methylation patterns from a single cell type in a mollusc, including an examination of the relationship between gene expression and promoter region methylation. Our results demonstrate that DNA methylation is predominant in intragenic regions (exons and introns) and that there is a positive relationship between methylation and gene expression in *C. gigas*. Furthermore, we were surprised to find similar patterns of tissue-specific methylation in male gametes as has been previously described in oyster gill tissue, thus suggesting that overall methylation levels do not dramatically vary between tissue types, and specifically between gametic and somatic cells.

## ***Methods***

### *Bisulfite treated DNA sequencing (BS-Seq)*

A single male adult oyster was collected from Thorndyke Bay, WA and thermally conditioned and fed for 6 weeks in the laboratory. Male gamete tissue was scored with a razor blade, gametes rinsed with sterile seawater, centrifuged, and immediately placed on dry ice then stored at  $-80^{\circ}\text{C}$  until further processing. Genomic DNA was extracted using DNAzol according to the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH). High molecular weight genomic DNA (6 ug) was used to prepare a library for whole-genome bisulfite sequencing. Lambda DNA (Promega Co. Madison,

WI) was added to the sample prior to fragmentation and library construction to serve as a measure of bisulfite conversion efficiency. Extracted DNA was fragmented to an average length of 250 bp using a Covaris S2 (Covaris Inc. Woburn, MA) and fragment size was confirmed by gel electrophoresis. The library was constructed using the Paired-End DNA Sample Prep Kit (Illumina, San Diego, CA) with standard protocols. DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA) and 72 bp paired-end sequencing was performed on the Illumina HiSeq 2000 system. Library construction and sequencing was performed by the High Throughput Genomics Center (htSEQ, Seattle, WA).

DNA sequence reads were mapped to all genomic scaffolds from the *Crassostrea gigas* draft genome (Fang et al., 2012). Sequences were mapped using Bisulfite Sequencing Mapping Program BSMAP v2.73 (Xi and Li, 2009). Resulting data from mapping bisulfite treated reads was analyzed with *methratio*, a Python script that accompanies BSMAP to calculate and extract methylation ratios. Parameters for *methratio* included reporting loci with zero methylation ratios (-z), combining CpG methylation ratios on both strands (-g) and only using unique mappings (-u). The same mapping procedure was also performed with the *Crassostrea gigas* mitochondrial genome (Accession # AF177226). The resulting *methratio* outputs were uploaded to SQLShare (Howe et al., 2011) and queried to examine distribution of methylation. Methylation characteristics were initially calculated for all cytosines.

#### *Genomic features and CpG dinucleotide methylation*

Methylation of CpG dinucleotides was characterized in relation to genomic features. A CpG locus was considered methylated if it had at least 5× coverage and at

least half the reads remained unconverted after bisulfite treatment. Methylation ratios were calculated for individual loci as well as for full-length genes and intragenic regions (introns and exons). Methylation on a per gene basis was determined by obtaining the number of methylated cytosines divided by the total number of CpG dinucleotides per region. Genome feature tracks were generated in order to characterize the distribution of methylation in the male gamete tissue. All CpG dinucleotides were identified using the EMBOSS tool fuzznuc (Rice et al., 2000). Methylated CpGs (5× coverage, ≥50% unconverted), sparsely methylated CpGs (5× coverage, 0–50% unconverted) and unmethylated CpG loci (5x coverage, 0% unconverted) within genomic regions were determined using Bedtools (i.e., *intersectBED*) (Quinlan and Hall, 2010). Methylation was examined within exons and introns (Fang et al., 2012), promoter regions (characterized as 1 kb regions upstream from transcription start sites), and putative transposable elements identified using RepeatMasker and the Transposable Element Protein Database (Smit et al., 1996-2010).

### *Transcriptome sequencing*

Total RNA was isolated using TRI reagent (Molecular Research Center) from the same oyster gamete tissue used for bisulfite sequencing. RNA was enriched for mRNA using Sera-Mag oligo dT beads (Thermo Scientific). A shotgun library was constructed from double stranded cDNA for paired end sequencing by end-polishing, A-tailing and ligation of sequencing adaptors. Sequencing and library preparation were performed on the Illumina HiSeq 2000 platform at the Northwest Genomics Center at the University of Washington (Seattle, WA). RNA-Seq analysis was performed using CLC Genomics Workbench version 6.5 (CLC Bio, Aarhus, Denmark) with high-throughput reads (50 bp

paired end) mapped back to the oyster transcriptome (Fang et al., 2012). Initially, sequences were trimmed based on quality scores of 0.05 (Phred; Ewing and Green, 1998; Ewing et al., 1998), and the number of ambiguous nucleotides (>2 on ends). Sequences smaller than 20 bp were also removed. For RNA-Seq analysis, expression values for each gene (28,027) were measured as RPKM (reads per kilobase of exon model per million mapped reads) (Mortazavi et al., 2008) with an unspecific match limit of 10 and maximum number of 2 mismatches.

A Chi-squared test was performed to determine if the degree of gene methylation with respect to gene expression levels (RPKM) was different from what would be expected from a random distribution of methylation levels in promoter regions ( $p$ -value < 0.05 was considered significant). For promoter region analysis, these regions were determined to be the 1 kb regions upstream from transcription start sites that did not overlap with neighboring genes. In addition, only promoter regions with at least 10 CpG dinucleotides were considered. Oyster genes were classified as either heavily methylated (methylation ratio  $\geq 0.5$ ), sparsely methylated (methylation ratio 0–0.5) or unmethylated (methylation ratio = 0).

## ***Results***

### *Bisulfite treated DNA sequencing (BS-Seq)*

Bisulfite treated DNA sequence reads (171.5 million) were produced and are available (NCBI Sequence Read Archive: accession number SRX386228). A total of 90 million paired end reads (53% of total reads) and 32 million single end reads (9.6%) mapped to the *Crassostrea gigas* genome. Sodium bisulfite conversion efficiency was

estimated to be 99.72% based on analysis of lambda phage DNA. All cytosine dinucleotide motifs were examined and a majority of methylated cytosines were reported in CpG dinucleotides. We found that 15% of the CpG dinucleotides were methylated while the next highest motif (CpA) methylated at 0.14%, which falls within the sodium bisulfite conversion efficiency margin of error (0.28%).

#### *DNA methylation and genomic features*

The bisulfite sequencing effort provided  $\geq 1x$  coverage for 8.52 million of the 9.98 million CpGs (85%) in the oyster nuclear genome. Using a 5x coverage threshold, which corresponds to 7.64 million CpGs (77%), the majority of CpGs were not methylated (Figure 1).

The proportion of CpG methylation occurring in specific regions of the oyster genomic landscape were characterized. Methylation occurs predominantly in intragenic regions, with 74% of methylated CpGs found in exons and introns. A total of 30% of CpGs in exons were methylated and 18% of CpGs in introns were methylated (Figure 2). These are particularly high levels of methylation when compared to methylation levels of other oyster genomic regions, wherein between 4 and 7% of CpGs were methylated.

The oyster mitochondrial genome is predominantly unmethylated. With an average coverage of 39.76-fold, of the 2518 cytosines with at least 5x coverage, 2316 cytosines were converted upon bisulfite treatment and no cytosines were considered methylated.

#### *Whole transcriptome sequencing expression patterns*

After quality trimming, 50.3 million reads (paired end 50 bp) remained (NCBI Sequence Read Archive: accession number SRX390346). Expression (RPKM) was

detected in a majority of the genes (17,093 genes or 63%). Median expression level was 0.749 and expression level ranged from 0 to 35637.3 RPKM. The relationship between gene methylation and expression levels was examined by determining methylation on a per gene basis.

A minimum of one methylated CpG dinucleotide with  $\geq 5\times$  coverage was observed for every 14,517 genes in the *C. gigas* genome, or 53% of genes. The proportion of methylated CpGs was characterized with respect to RNA-Seq data on expression levels for full-length genes. Specifically, within genes and putative promoter regions we found a greater proportion of fully methylated CpGs for genes that have elevated expression levels ( $>1$  RPKM) (Figure 3). The observed distributions of methylated CpGs within genes ( $X^2 = 5493.85$ ,  $df = 2$ ,  $p < 0.0001$ ) and promoter regions ( $X^2 = 1765.56$ ,  $df = 2$ ,  $p < 0.0001$ ) were significantly different than what would be expected if methylated CpGs were randomly distributed among genes and promoter regions.

Additionally, our transcriptome data was compared to a comprehensive microarray experiment focused on gametogenesis in *C. gigas* (Dheilly et al., 2012). Elevated gene expression was observed in genes identified by Dheilly et al. (2012) to increase in expression over spermatogenesis (clusters 5 and 6) (Figure 4). Based on this analysis, we suspect our sample was likely from a fully mature male gonad.

### ***Discussion***

Genome-wide shotgun bisulfite sequencing was conducted on gametes from a male oyster to illustrate the role of DNA methylation in a single cell type. While reduced representation CpG DNA methylation has been previously quantified for this species, this

study represents the first examination of other dinucleotide motifs. Although CpG DNA methylation is ubiquitous across organisms, types and levels of methylation vary considerably among invertebrates. For example, *Caenorhabditis elegans* essentially lacks DNA methylation in its genome where neither 5-methylcytosine nor DNA methyltransferase are present (Bird, 2002). The *Drosophila melanogaster* genome contains extremely low 5-methylcytosine levels (Gowher et al., 2000; Lyko et al., 2000) and mostly in the CpT dinucleotide context rather than CpG (Lyko et al., 2000). In this study, DNA methylation was only observed in CpG motifs.

This is the first characterization of DNA methylation in bivalve mitochondrial DNA. A lack of methylation in oyster mitochondrial DNA in our study is consistent with previous invertebrate research, which found that genes encoding mitochondrial DNA were unmethylated in the brain tissue of the honey bee *Apis mellifera* (Lyko et al., 2010). Recent research has also demonstrated the absence of CpG methylation in the mitochondrial genome of the sea squirt *Ciona intestinalis* (Suzuki et al., 2013). Studies on the mammalian system have found similar results, confirming a lack of methylation among CpGs in human mitochondrial DNA (Hong et al., 2013).

Overall, approximately 15% of the CpGs are methylated in the oyster male gamete nuclear genome. This is the same estimated proportion from analysis of DNA methylation in oyster gill tissue (Gavery and Roberts, 2013). Similarly, (Suzuki et al., 2013) found identical gene groups to be methylated in tissues from *C. intestinalis* sperm and muscle cells. In their study 23% of the genome was determined to be methylated (Suzuki et al., 2013). The degree of methylation we found in oyster male gamete tissue falls within those previously described for other molluscs. For instance, recent research

examining DNA methylation in multiple tissues of the mollusc *Chlamys farreri* found methylation levels in the genome to be around 21% using a methylation-sensitive amplification polymorphism approach (Sun et al., 2014). Studies of DNA methylation in the foot tissue of the gastropod *Biomphalaria glabrata* found approximately 2% of the CpG dinucleotides to be methylated (Fneich et al., 2013). Our findings, in agreement with previous studies in *C. gigas*, corroborates that overall genome methylation in *C. gigas* is at an intermediate level and suggests that DNA methylation does not significantly vary among tissue type.

DNA methylation is predominantly found in exons and introns in oyster male gametes (Figure 2). These findings are consistent with previous work characterizing DNA methylation in *Crassostrea gigas* gill tissue (Gavery and Roberts, 2013). In several animal and plant genomes, transcribed regions of genes, including intragenic regions, have higher levels of DNA methylation than neighboring regions (Suzuki et al., 2007; Zilberman et al., 2007). DNA methylation in insects, however, appears to be primarily confined to exons (Lyko et al., 2000). Recent work examining DNA methylation in *C. intestinalis* sperm found that methylated domains of the genome are primarily contained within transcription units and promoter regions, with intergenic regions completely unmethylated (Suzuki et al., 2013). Together these studies demonstrate that the distribution of DNA methylation in invertebrate genomes is diverse and that most invertebrate genomes exhibit interspersed regions of methylated and unmethylated DNA. Although we have a relatively comprehensive profile of where DNA methylation occurs across the oyster genome, there is no definitive evidence of a link between DNA

methylation and function. However, based on RNA-Seq data there is likely an association between methylation status and gene expression in *C. gigas*.

Recently, Riviere et al. (2013) proposed that proximal promoter and first exon methylation in *C. gigas* larvae have similar functions than those in mammalian systems, where increased methylation in promoter regions corresponds to decreased gene expression. Specifically, Riviere et al. (2013) reported a negative correlation between DNA methylation and expression of some *homeobox* gene orthologs during early oyster development. Our results indicate the opposite- a positive correlation between DNA methylation and gene expression (Figure 3), in which genes with high expression levels had high methylation levels. This is similar to what has been reported for oyster gill tissue (Gavery and Roberts, 2013). Riviere et al. (2013) suggested that a lack of DNA methylation influences gene expression specifically by controlling the transcription level of *homeobox* orthologs in the proximal promoter and first exon. Similarly, the presence of promoter methylation has specifically been associated with transcriptional silencing among many organisms (Suzuki and Bird, 2008). Here we found a significant difference in promoter methylation in high versus low gene expression. It is likely that the relationship of DNA methylation and gene expression is complex and dependent on several factors (Gavery and Roberts, 2014). For example, it has been shown that methylation pattern is dependent on gene function (Roberts and Gavery, 2012) and the current study focused on the entire transcriptome where Riviere et al. (2013) specifically looked at *homeobox* orthologs. The differences we observed between DNA methylation and expression than was previously reported (Riviere et al., 2013) could also be attributable to differences in cell type or a differing role of methylation throughout *C.*

*gigas* life history stages. It should also be noted both studies have limitations as they rely on accurate genome annotation, and, as recently reported (Elsik et al., 2014), this can significantly alter research findings.

Here we provide the first genome-wide characterization of DNA methylation in a bivalve that focused on a single-cell type. We found that DNA methylation levels in male gametes are similar to those previously reported in gill tissue (Gavery and Roberts, 2013). In examining the relationship between DNA methylation and transcript expression, we observed a pattern suggesting that promoter regions with a higher proportion of CpG methylation are associated with highly expressed genes. Further explorations into the roles of miRNA and histone modifications will help to elucidate our understanding of epigenetic regulatory functions in oysters.

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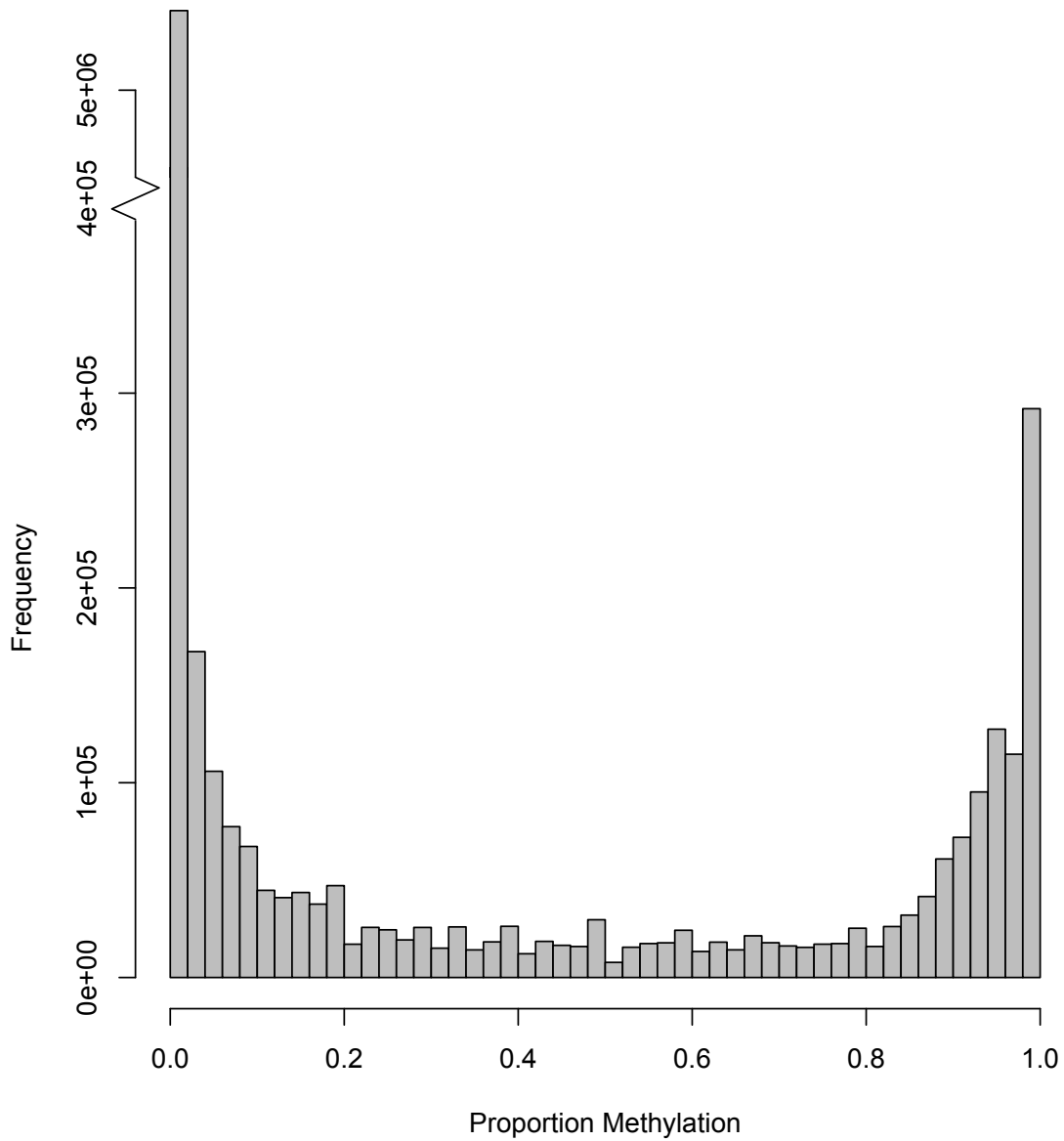
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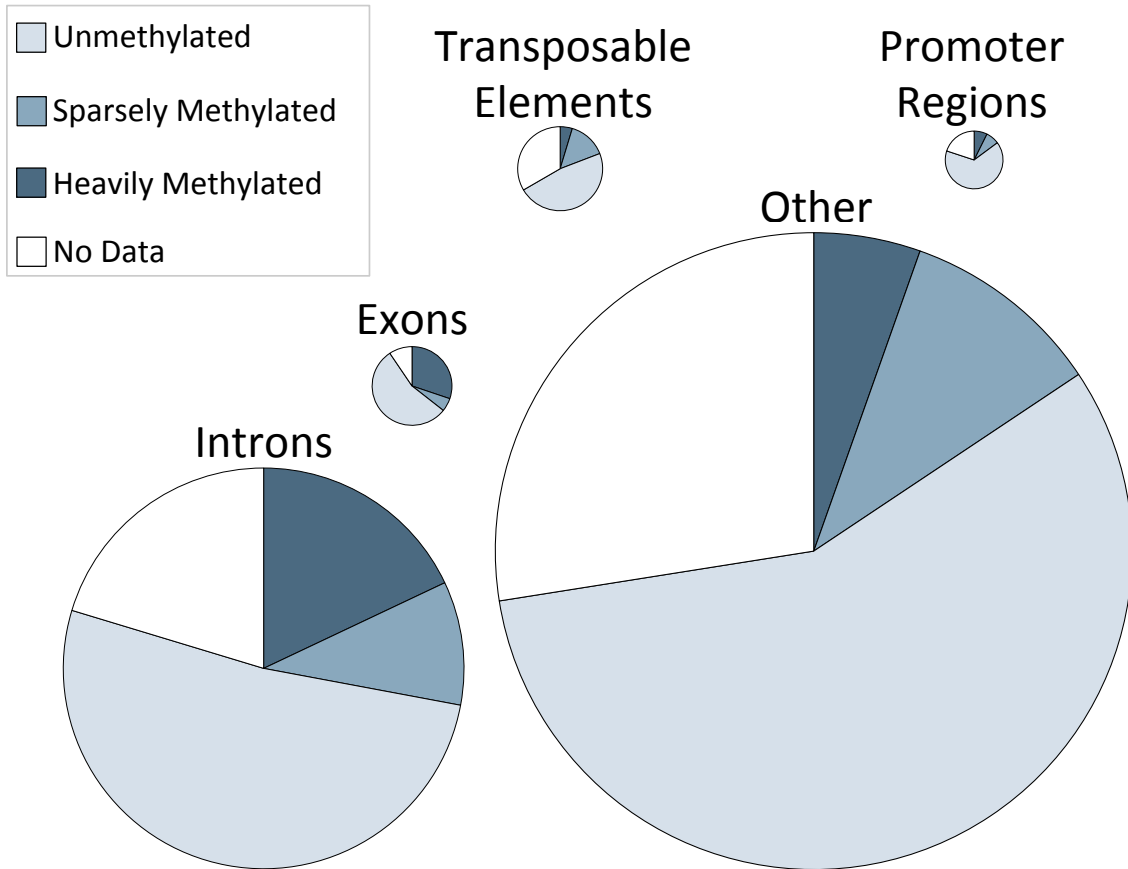
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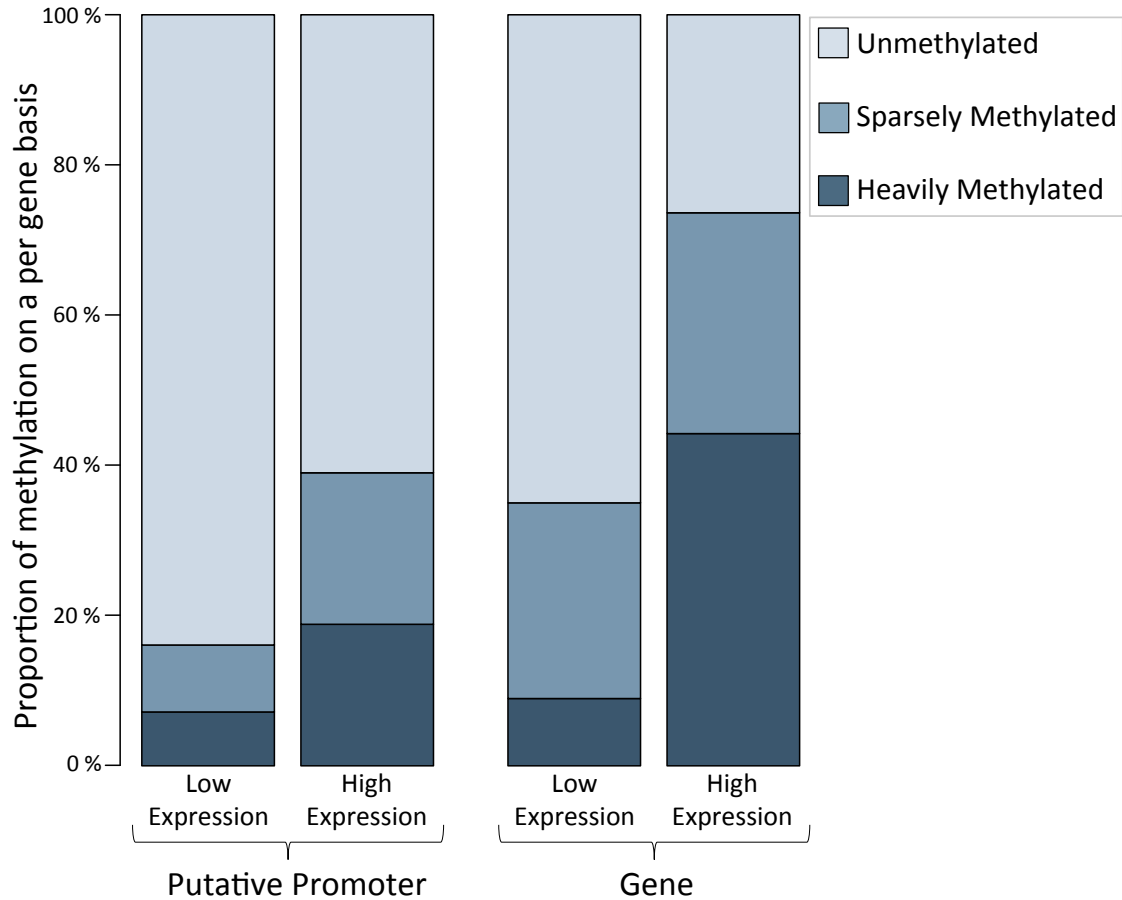
**Figures**



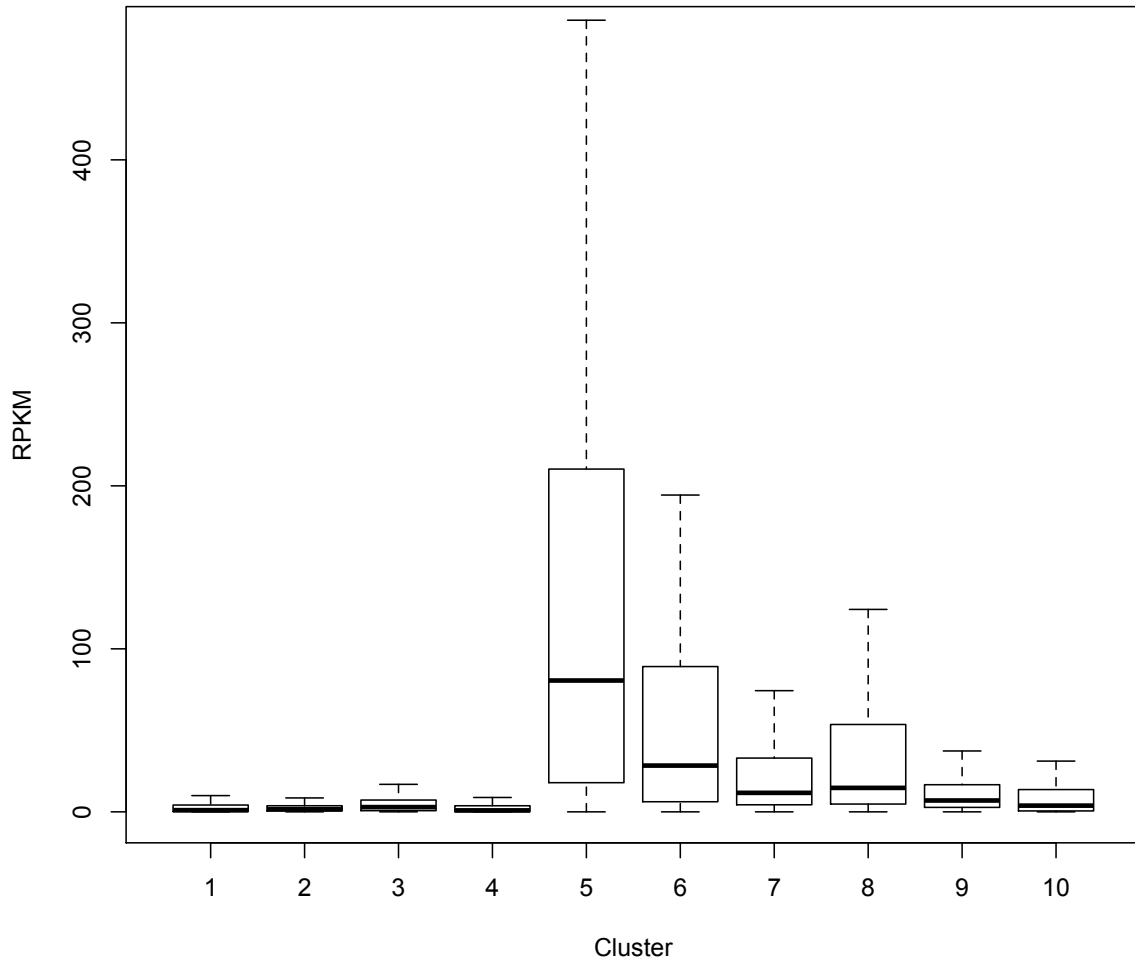
**Figure 1.** Frequency of CpG methylation ratios in *C. gigas* male gamete tissue. A total of 7,642,816 CpG dinucleotides with at least 5x coverage were examined.



**Figure 2.** Genome-wide distribution of CpG methylation in *C. gigas* male gamete tissue within genomic regions. The proportion of CpG unmethylated (ratio = 0), sparsely methylated (ratio between 0 and 0.5), heavily methylated (ratio  $\geq 0.5$ ), or CpG dinucleotides with less than 5x coverage (“No data”) are shown as percentage contributions to specific oyster genomic regions. Genomic regions are scaled according to their relative CpG contribution to the genome.



**Figure 3.** Proportion of methylation on a per gene basis for putative promoter regions and gene bodies (exons and introns) for high and low expression levels indicate that DNA methylation is positively correlated to gene expression in *C. gigas* male gamete tissue. Two classifications shown include genes with low expression (Promoter regions = 13,919 and genes = 14,377; RPKM  $\leq 1$ , including no expression) and high expression (Promoter regions = 12,477 and genes = 12,877; RPKM  $> 1$ ). The proportion of corresponding genes that are unmethylated (methylation ratio = 0), sparsely methylated (methylation ratio 0-0.5), and heavily methylated (methylation ratio  $\geq 0.5$ ) are shown.



**Figure 4.** Boxplots of expression levels (RPKM) from RNA-Seq data relative to the 10 clusters of similar gene expression patterns during oyster gonad development identified by Dheilly *et al.* 2012. The number of genes represented are 511 (Cluster 1), 222 (Cluster 2), 312 (Cluster 3), 197 (Cluster 4), 226 (Cluster 5), 146 (Cluster 6), 200 (Cluster 7), 295 (Cluster 8), 332 (Cluster 9), and 41 (Cluster 10). For a full description and all files please see:

Olson C., Roberts S. (2014). *Crassostrea gigas* male gonad transcriptome data comparison. Figshare. <http://dx.doi.org/10.6084/m9.figshare.1004464>

## **Chapter II: Indication of family-specific DNA methylation patterns in developing oysters**

### ***Abstract***

The roles of DNA methylation during invertebrate development remain enigmatic, especially regarding the inheritance and ontogenetic dynamics of methylation. Here, we characterized the genome-wide methylome of *Crassostrea gigas* sperm and larvae from two full-sib families nested within a maternal half-sib family across several developmental stages. Our data suggest that DNA methylation patterns are inherited, as methylation patterns were similar between the two sires and their offspring. Loci differing between the two paternal full-sib families (189) and among the developmental stages (160) were found throughout the genome but were concentrated in transposable elements and repeat regions. We suggest that the predominance of differentially methylated loci within transposable elements is a result of selection against changes in gene body methylation.

### ***Introduction***

DNA methylation is an epigenetic modification that is ubiquitous across many eukaryotes, with variable patterns and functions across taxa. This epigenetic mechanism involves the addition of a methyl group to cytosines, usually in CpG dinucleotides, catalyzed by DNA methyltransferases. Epigenetic modifications such as DNA methylation can alter gene expression without modifying the underlying nucleotide sequence, and functions in mammals to suppress transcription through increased methylation in promoter regions (Bell and Felsenfeld, 2000). In mammals, DNA methylation is essential for development and differentiation of organs and tissues (Okano

et al. 1999). Likewise, mutations of DNA methyltransferase in mammals may result in developmental delays and mortality (Li et al. 1992).

In contrast to the densely methylated mammalian genomes, several invertebrate species display low to intermediate levels of methylation. In invertebrates, it has been proposed that DNA methylation of genes may be associated with alternative splicing events (i.e. honey bee (Lyko et al. 2010) and *Nasonia* (Park et al. 2011)). Methylation of gene bodies has also been shown to have a positive relationship with transcriptional activity in oysters (Gavery and Roberts, 2013; Olson and Roberts, 2014a). Currently there is an incomplete understanding of the regulation of gene expression by DNA methylation in invertebrates, though it appears to be distinct from mechanisms observed in mammals and likely varies across species.

From the limited studies that have focused on invertebrates, research has shown that similar to mammals, DNA methylation has important regulatory functions during early development. For example, research on the honey bee *Apis mellifera* found DNA methylation to be abundant in the genome, with methylation being associated with altered gene expression resulting in bee caste differentiation (Elango et al. 2009; Kucharski et al. 2008). Furthermore, DNA methylation has been shown to regulate gene expression during *Octopus vulgaris* development, particularly during the first paralarval stage that includes significant morphological changes (Diaz-Freije et al. 2014). The first indication that methylation was an important regulator of development in *C. gigas* was by Riviere et al. (2013), who found treatment with 5-Aza-cytidine leads to developmental alterations and abnormal phenotypes in oysters.

Despite the essential role of methylation in development, there is limited information on individual variation in DNA methylation patterns among invertebrates and particularly how any methylation information might be passed on to offspring. Furthermore, we do not have a full understanding of ontogenetic changes in DNA methylation. In order to better understand to what degree DNA methylation patterns are heritable, variable between individuals, and changing during *C. gigas* development, we analyzed genome-wide DNA methylation in gametes and larval oysters (72 and 120 hours post-fertilization) from two full-sib families.

## ***Methods***

### *Experimental Design*

Oysters (two males and a single female) were collected from Oakland Bay, South Puget Sound, WA. Oysters were strip spawned by scoring the gonad tissue with a sterile razor blade and rinsing out gametes with sterile seawater. Oocytes were incubated for 30 minutes in sterile seawater and 2 million oocytes each were placed into two separate plastic containers. Sperm diluted in sterile seawater (1L) from each male were used to fertilize oocytes. Fertilization was confirmed by examining polar bodies in cells under a compound microscope.

Larvae were kept in static tanks (100L) up to 120 hours post-fertilization (hpf). Counts of oyster larvae were performed at 120 hpf to confirm normal development. Two samples for DNA methylation analyses were taken from sperm prior to fertilization, and larvae samples collected at days 72 hpf and 120 hpf. Larvae samples were taken by filtering on a 20 $\mu$ m screen. All samples were preserved in 95% ethanol.

For simplicity the sperm and corresponding larvae samples are referred to as family #1 and family #3 based on paternity.

#### *Bisulfite treated DNA Sequencing (BS-Seq)*

Genomic DNA was extracted using DNAzol according to the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH). High molecular weight genomic DNA (6 ug per sample), was used to prepare six libraries for whole-genome bisulfite sequencing. Briefly, DNA was fragmented to an average length of 250 bp in an Adaptive Focused Acoustics (AFA) microtube using a Covaris S2 (Covaris Inc Woburn, MA) with the following settings: duty cycle 20%, intensity of 4.0, cycles per burst 200, for 60 seconds. Fragment size resulting from DNA shearing was confirmed by gel electrophoresis. Libraries were constructed using the Paired-End DNA Sample Prep Kit (Illumina, San Diego, CA) with standard protocols. Unmethylated Lambda DNA (0.5%) (Promega Co. Madison, WI) was added to the each sample prior to fragmentation and library construction to serve as a measure of bisulfite conversion efficiency. DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA) and 72 bp paired-end sequencing was performed on the Illumina HiSeq 2000 system. Library construction and sequencing was performed by the High Throughput Genomics Center (htSEQ, Seattle, WA).

Bisulfite sequencing reads from the six libraries were quality filtered to remove adapter sequences and separately mapped to the *Crassostrea gigas* genome (version GCA\_000297895.1; Zhang et al. 2012) using Bisulfite Sequencing Mapping Program BSMAP v2.74 (Xi and Li 2009). Resulting alignment from mapping bisulfite treated reads was analyzed with *methratio*, a Python script that accompanies BSMAP.

Parameters for *methratio* included reporting loci with zero methylation ratios (-z), combining CpG methylation ratios on both strands (-g) and only using unique mappings (-u). Only CpG loci covered by at least 3 sequenced reads were considered for further analysis. Data can be accessed and computational analysis performed as described via a GitHub repository (Olson and Roberts, 2014b). The IPython notebook in this repository includes all steps necessary for downloading and reproducing the analyses described in this manuscript.

#### *Global DNA Methylation Comparison*

Whole-genome DNA methylation correlation and clustering were performed using the program methylKit 0.9.2 (Akalin et al. 2012) in R v3.0.3. Pairwise Pearson's correlation coefficients scores were calculated based on the percent methylation profiles between all pairs of samples. Hierarchical clustering was performed using 1-Pearson's correlation distance of the six methylation profiles.

#### *Differentially Methylated Loci*

Differential methylation between the two full-sib families at each locus was determined using Fisher's exact test in methylKit. A CpG locus was determined to be different between families when the difference in methylation ratio between families was more than 25% and  $p$ -value  $<0.01$ .

Ontogenetic changes in DNA methylation patterns were tested by three pairwise comparisons (Fisher's exact tests in methylKit) between all developmental stages (sperm and 72 hpf larvae, sperm and 120 hpf larvae, and 72 hpf and 120 hpf larvae).

Differentially methylated loci were identified as any CpG with greater than 25% and  $p$ -value  $<0.01$  for any comparison.

All loci with significantly different methylation ratios across families and ontogenetic stages were characterized with respect to genomic features (intron, exon, promoter region, transposable element) using Bedtools (i.e., intersectBed) (Quinlan and Hall, 2010). Genomic features were developed and reported elsewhere (Gavery and Roberts 2013). Putative promoters were defined as 1kb regions upstream from transcription start sites. Transposable elements were identified using RepeatMasker, a program that screens and annotates interspersed repeats (Smit et al., 1996-2010). Specifically, RepeatProteinMask, was used with rebase which contained 7,445 peptide sequences. RepeatProteinMask also uses Tandem Repeat Finder (Benson, 1999) to identify tandem repeats which were included in the genome feature track. A total of 119,786 features are in the transposable element genome feature file used for analysis in this paper including 61,319 tandem repeat regions and 58,467 transposable elements identified based on sequence similarity. This genome feature track along with intron, exon, and promoter region feature track are all available via the IPython notebook that provides code and data used in this manuscript (Olson and Roberts, 2014b). A Chi-squared test was performed to determine if the distribution of differentially methylated loci among genomic regions (intron, exon, promoter region, transposable elements) was significantly different to the distribution of all CpGs in the oyster genome.

## ***Results***

### *Bisulfite treated DNA Sequencing (BS-Seq)*

Bisulfite treated DNA sequence reads are available (NCBI Sequence Read Archive: Study Accession Number SRP028178 - Accession Numbers SRX795174-SRX795179). Sodium bisulfite conversion efficiency was estimated to be 99.9% based

on analysis of lambda phage DNA. The number of sequenced cytosines ranged from  $2.6 \times 10^7$  to  $5.3 \times 10^7$  across libraries. Using a 3x coverage threshold, most cytosines (75-78%) were determined to be unmethylated (methylation ratio = 0), while 15-18% of the CpG dinucleotides were methylated (methylation ratio  $\geq 0.5$ ) (data not shown).

#### *Genome-wide DNA Methylation Comparison*

Relationships on a genome-wide scale were assessed by sample correlation and clustering. A total of 40,654 common loci were shared among all six samples and thus analyzed. Methylation ratios were highly correlated between sperm and respective progeny, with a pair-wise Pearson's correlation coefficient ( $r$ ) of  $\geq 0.84$  for both families. These similarities within families are also evident in hierarchical clustering (Figure 1), where both male gamete samples were more similar in their methylation profiles to their respective 120 hpf larvae.

#### *Family-specific and Developmental Differences*

A total of 189 differentially methylated loci (DMLs) were identified between the two full-sib families. Of these, 102 were found to overlap with a defined genomic region (exon, intron, promoter region, transposable element) (Figure 2). Most CpG loci with different methylation ratios among oyster families were in introns. However, compared to the distribution of CpG dinucleotides in the oyster genome, the proportion of differentially methylated loci within transposable elements was significantly higher than expected ( $\chi^2 = 18.84$ ,  $df = 1$ ,  $p < 0.0001$ ).

A total of 160 CpG loci showed differences in methylation ratios among developmental stages. Of these loci, 99 were within defined genomic regions (Figure 2).

Again, the proportion of differentially methylated loci was significantly greater in transposable elements than in the rest of the oyster genome ( $\chi^2= 9.76$ ,  $df= 1$ ,  $p < 0.0018$ ).

### ***Discussion***

This study provides the first single-base pair resolution DNA methylomes for both oyster sperm and larval samples from multiple crosses. This research not only provides new information on DNA methylation patterns during oyster development, but also examines its inheritance and changes during early development. Interestingly, our research indicates that epigenetic patterns may differ among oyster families.

Methylation levels in oyster sperm and larvae samples ranged from 15-18% with interspersed regions of both methylated and unmethylated DNA in both male gamete and larval samples. This proportion of CpG methylation falls within the range of that previously reported for oyster male gonad tissue (Olson and Roberts, 2014a) and oyster gill tissue (Gavery and Roberts, 2013). Overall methylation levels are also comparable to those reported among multiple developmental stages of the Pearl oyster *Pinctada fucata* (Li et al. 2014). These findings indicate that overall genome methylation in *C. gigas* is at an intermediate level and suggests that DNA methylation levels do not significantly vary between multiple cell types and life history stages. This is similar to what has been described in global 5-methylcytosine content during different stages of *Ciona intestinalis* development (Suzuki et al. 2013). However, it contrasts with research on mammals and vertebrates, which exhibit the presence of tissue and developmental stage specific methylation profiles, as that seen in zebrafish (McGaughey et al. 2014).

Genome-wide comparisons indicated higher similarity of methylation patterns between oyster families than between developmental stages, suggesting that DNA

methylation patterns are inherited. If epigenetic marks are indeed heritable, this mechanism has significant implications for selection. It has been proposed that epigenetic variation may compensate for a decrease in genetic variation in species such as sparrows (Shrey et al. 2012). While outside the scope of the current study, an assessment of relationships between genetic and epigenetic variation is critical. Several studies have examined epigenetic differentiation in vertebrate and plant populations experiencing different environments, indicating evidence for divergent selection in these species (Liu et al. 2012; Herrera and Bazaga 2010). Few studies have focused on invertebrates, though Jiang et al. (2013a) investigated the relationship between genetic and epigenetic variations in two groups of *C. gigas*, a base stock domesticated population and third generation mass selection population. This study demonstrated genetic differentiation between the base population and third generation mass selection populations of oysters, but did not find overall epigenetic variation (Jiang et al. 2013a). Nevertheless, a significant correlation was observed between genetic and epigenetic profiles, with few individuals having similar genetic but distinct epigenetic profiles (Jiang et al. 2013a). Regardless, if epigenetic variation is independent of genotype, mechanisms involved in epigenetic inheritance are not fully understood.

Differences in genome-wide methylation ratios between full-sib families nested within a maternal half-sib family suggested paternal inheritance of DNA methylation patterns. These results are similar to what has been seen in zebrafish where embryos inherit the methylation profile of sperm rather than oocyte (Jiang et al. 2013b, Potok et al. 2013). In contrast, methylation ratios in Pearl oysters are mainly influenced by oocytes, rather than sperm (Li et al. 2014), possibly due to maternal influences on DNA

methylation patterns in early larvae, while later stages attain methylation patterns similar to sperm.

Differentially methylated loci across families were distributed throughout the genome, through a higher proportion was found in transposable elements. This concentration of methylation in transposable elements may be due to selection against methylation in functionally important parts of the genome. For instance, many differentially methylated loci in gene bodies could be lethal or deleterious as they would alter gene expression. It should be noted that the role of DNA methylation in regulating genome activity in *C. gigas* is still unclear. However, it has been suggested that elevated methylation decreases spurious transcription of housekeeping genes and limited methylation in inducible genes facilitates multiple transcriptional opportunities (Roberts and Gavery, 2012). In other words, DNA methylation patterns in gene bodies may have evolved over time based on gene function to fit the needs of organisms in highly variable environments, and random changes in these patterns could be detrimental. Furthermore, we suggest that random variations in methylation within transposable elements may have a relatively higher chance of persisting than elsewhere in the genome. Transposable elements are mobile DNA sequences that may be methylated in many species to silence activity (Yoder et al. 1997; Liu and Schmid 1993). Limited information is available about the methylation status of transposable elements in other invertebrate species, but the available studies suggest that transposons are generally unmethylated and contain similar levels of methylation to neighboring DNA (Suzuki and Bird, 2008). This is in agreement with our previous research, which showed limited DNA methylation in transposable elements in oyster male gamete tissue (Olson and Roberts, 2014a). Assuming that

transposable element activity is less critical to survival than coding gene activity, differentially methylated loci in transposable elements may be less likely to have negative selective effects. On the other hand, differentially methylated loci may also provide advantageous phenotypic variation by increasing transposable element mobility. However, such selection hypotheses assume that methylation is introduced randomly, something we do not have evidence for.

A significant proportion of loci differentially methylated during oyster development were found within transposable elements, relative to the distribution of CpG dinucleotides in the oyster genome. Interestingly, we did not observe a high proportion of differentially methylated loci among promoter regions, as would be expected if promoter methylation was regulating gene expression to play a role in oyster development. Recent research has found that DNA methylation of promoter regions specifically reduces expression of *Hox* genes during oyster development (Riviere et al. 2013). Considerable stage-specific differences in total methylation levels during oyster early development indicated that DNA methylation plays a crucial role in oyster embryogenesis (Riviere et al. 2013). We previously found variation in expression levels depending on the level of promoter region methylation (Olson and Roberts, 2014a). Surprisingly, we did not observe any dramatic differences in overall methylation levels during oyster development, nor higher methylation of promoter regions. This discrepancy is likely due to the analysis of different ontogenetic stages, as Riviere et al. (2013) examined the first 24 hours post-fertilization, and our first larval sample was taken at 72 hpf. It is also possible that only a subset of genes are transcriptionally controlled via DNA methylation and our global approach masked the ability to see differences.

In conclusion, this research suggests epigenetic inheritance as DNA methylation patterns were similar between males and their offspring and differed between oyster families. Interestingly, we found a high proportion of family-specific and stage-specific methylation patterns within transposable elements. Future research should focus on the relationship between epigenetic and genetic variation, and explore the possible relationship of DNA methylation and transposable element activity.

### ***Acknowledgements***

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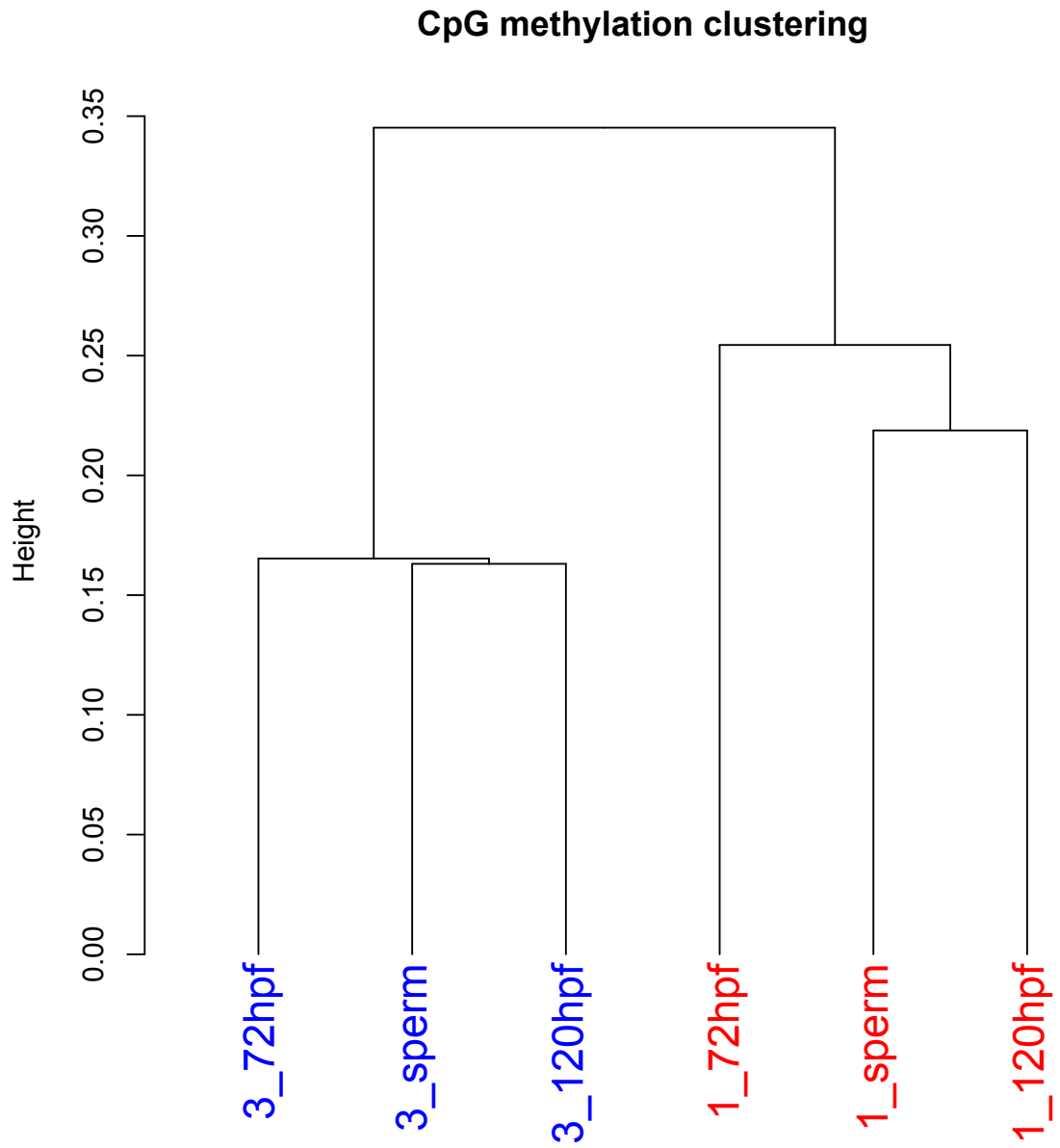
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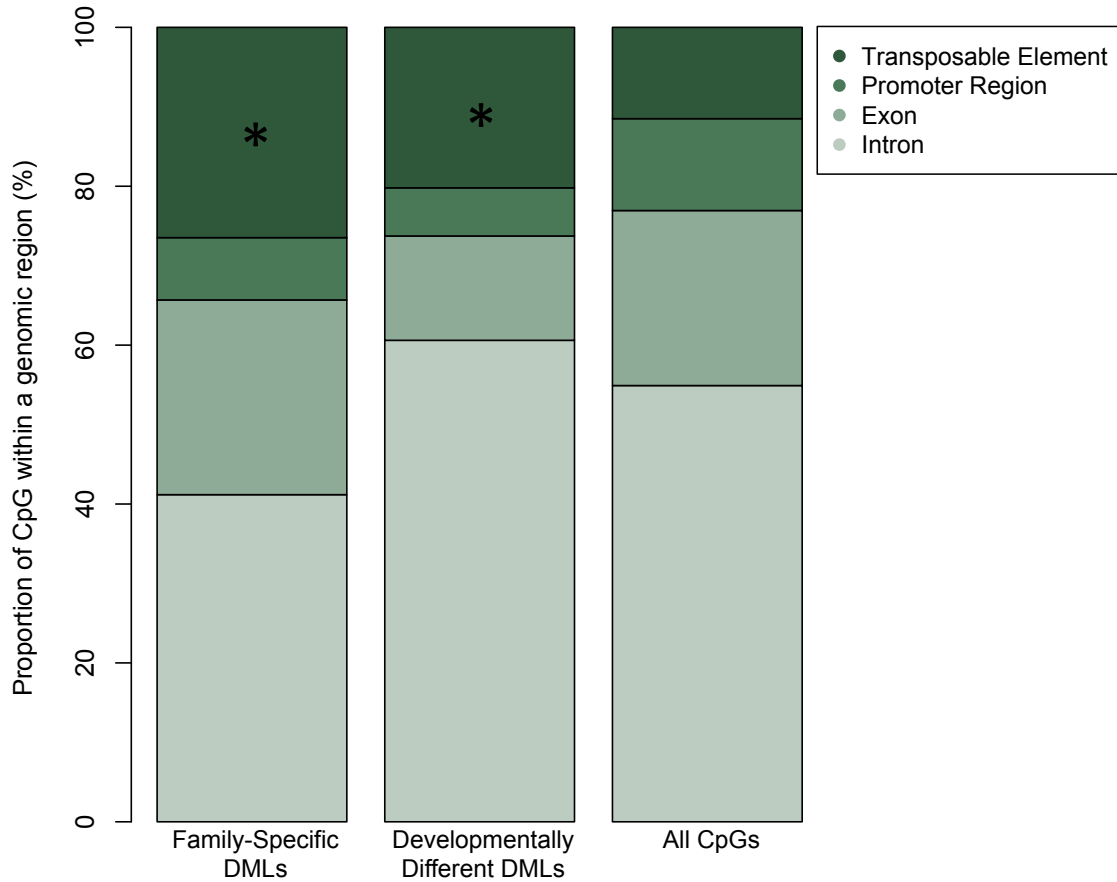
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**Figures**



**Figure 1.** Dendrogram of the male sperm and oyster larvae genome-wide methylation profiles using Pearson's correlation distance. Numeric prefix refers to family.



**Figure 2.** Proportion of family-specific differentially methylated loci, developmentally different differentially methylated loci, and all CpGs in the oyster genome based on genomic region. The proportion of loci located within a genomic region (Intron, Exon, Promoter Region, Transposable Element) for differentially methylated loci between families (n= 102), differentially methylated loci among the developmental stages (n= 99), and all CpGs in the oyster genome (n= 10035701) are displayed. An asterisk indicates a statistically different distribution relative to the distribution of all CpGs in the oyster genome.