Genome-wide analysis of DNA methylation reveals selection signatures of the grass carp during domestication

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ABSTRACT

With the rapid development of aquaculture, many fish species are domesticated and brought into cultivation. In the process of domestication, the domesticated fish undergone intense selection pressures and develop some adaptations and phenotypic traits, namely selection signatures, such as growth and metabolism, immunity, foraging and learning behaviors. However, how this selection signatures emerges is still not clear and the knowledge of molecular epigenetic mechanisms underlying selection signatures in fish is still in its infancy. Thus, we used a farmed fish, grass carp (*Ctenopharyngodon idellus*), as model species to detect these selection signatures and identify the candidate differentially methylated genes that are closely associated with these selection signatures at the level of whole genome, investigating the role of DNA methylation in the emergence of selection signatures during domestication. Our results showed that domesticated grass carp demonstrated four selection signatures, including growth and metabolism, immunity, foraging and learning behaviors, and 38 candidate genes were found associated with these traits. 16 genes are significant candidate genes which play major roles in the growth and metabolism, such as IGF-1 , GK , GYS1, etc. 11 genes are related to immunity, including . The GRM1, TAS1R1 and TAS1R3 genes are essential for the adaptation of domesticated grass carp to commercial feed in artificial rearing condition. The C-FOS, POMC and CBP genes may be responsible for the acquisition of novel feeding habits and contribute to faster growth indirectly by enhancing food intake. The findings here in will provide new insights to expand our understanding about the role of epigenetic modifications in shaping physiological phenotypes in this and other teleost models, which can contribute to efficient breeding of aquaculture stocks and restocking programmes.

Keywords:

DNA methylation

grass carp

selection signatures

66 **1. Introduction**

67 As an economically important food source of humans, fish is one of the most important
68 domesticated species (Teletchea ,et al. 2012). In modern agricultural industry, fish have undergone
69 strong long-term artificial selection and developed a range of adaptations and phenotypic traits,
70 namely selection signatures (Milla et al.,2021), such as higher flexibility in diet, rapid growth, less
71 stress susceptibility, a more socially tolerant disposition and enhanced prolificacy (Pasquet et al.,
72 2018), which may distinguish those domesticated breeds from their wild counterparts. These
73 phenotypic differences make fish highly suitable for animal agriculture and comparative studies.

74 Epigenetic changes is increasingly recognized to contribute to the emergence of phenotypic
75 differences (Goldberg et al., 2007). DNA methylation, the most widely studied epigenetic
76 regulatory mechanisms, can respond to environmental change and, at the same time, be stable
77 enough to be maintained throughout lifetime, even across generations (Anastasiadi et al., 2019).
78 DNA methylation has been associated with many biological process, including gene expression
79 regulation, genomic imprinting, X chromosome inactivation, embryonic development, the
80 alteration of chromatin structure and transposon inactivation, etc (Wilson et al., 2012; Jones et al.,
81 2012). Multiple approaches have been developed to analyze DNA methylation profiles at the
82 genome-wide level, including comprehensive high-throughput arrays for relative methylation
83 (CHARM), methylated DNA immunoprecipitation (MeDIP), methylation array,
84 reduced-representation bisulfite sequencing (RRBS), whole-genome bisulfite sequencing (WGBS),
85 single-cell reduced-representation bisulfite sequencing (scRRBS), single-cell whole-genome
86 bisulfite sequencing (scWGBS), TET-assisted bisulfite sequencing (TAB-seq), etc (Laird et al.,
87 2010, Yong et al., 2016). Compared to other approaches, WGBS has two major advantages: (1) it
88 is able to assess the methylation state of nearly every CpG site, including low-CpG-density

89 regions, such as intergenic 'gene deserts', partially methylated domains and distal regulatory
90 elements, (2) it can determine absolute DNA methylation level and reveal methylation sequence
91 context (Yong et al., 2016). This method has been broadly employed to analyze the genome-wide
92 methylation profiles of many animals, including sea cucumber (Yang et al., 2020), pig (Yang et al.,
93 2016), broilers (Hu et al., 2013), Chinese perch (Pan et., 2021).

94 In recent years, several studies have focused on the role of DNA methylation regulation on
95 fish during domestication. However, most studies focus on the role of DNA methylation in the
96 changes of one particular phenotypic characteristics, such as growth (Li et al., 2017), reproduction
97 (Tomasz Podgorniak et al., 2019), morphological changes (Zhang et al., 2017), learning behaviors
98 (Dou et al., 2018). Thus, until now, no systematic studies have investigated the role of DNA
99 methylation in the emergence of different selection signatures in the process of fish domestication,
100 including growth and metabolism, immunity, foraging and learning behaviors.

101 Grass carp (*Ctenopharyngodon idellus*) is one of the most important freshwater aquaculture
102 species, and its global production reaches 5.704 million tons in 2018, the highest in fish
103 production worldwide, providing low-cost, high-quality animal protein especially for developing
104 and underdeveloped regions (FAO, 2020). Although some farms have carried out breeding for
105 several generations, others still rely on wild-caught fish for broodstock to maintain genetic
106 diversity (Huang et al., 2015; Fu et al, 2015). In addition, farmed grass carp also shares some of
107 the characteristics of the domestication syndrome, for instance, changes in morphological features
108 (Zhao et al., 2020), rapid growth (Ashraf et al., 2011), anti-predator behavior (Tang et al, 2017),
109 indicate that this species could be a suitable model species for studying the role DNA methylation,
110 one of key molecular epigenetic pathways, in the formation of selection signatures in this and

111 other teleost species.

112 In this study, we used WGBS to carry out genome-wide DNA methylation analysis of blood
113 from domesticated and wild carp with four selection signatures differences: growth and
114 metabolism, immunity, foraging and learning behaviors. We obtained comprehensive DNA
115 methylation profiles for the two groups and identified differentially methylated genes (DMGs) that
116 might contribute to the emergence of selection signatures between domesticated and wild carp. To
117 the best of our knowledge, this study is the first one to report the role of epigenetic modifications
118 in the emergence of different selection signatures in fish. Thus, the findings here in will provide
119 new insights to expand our understanding about the role of epigenetic modifications in shaping
120 physiological phenotypes in this and other teleost models

121

122 ## 2. Materials and Methods

123 *2.1 Domesticated trial*

124 A total of 15 wild sub-adult grass carp (initial weight 250 ± 20 g) were captured using traps
125 and gill nets along the Xijiang river in Zhaoqing, Guangdong Province, China, within a 1000 m
126 radius from the following coordinates (latitude: 23.1034.8 °N; longitude: 112.4554.0 °E).
127 Subsequently, the fish were transported to breeding base and randomly allocated into three ponds
128 (1.5 m × 1.5 m × 1 m), 5 individuals in each pond for subsequent domesticated experiments in the
129 Pearl River Fisheries Research Institute. The fish were fed commercial feed at 8:00 am and 4:00
130 pm each day for 180 days, and the feed amount for each day was 2~3% of fish weight. The water
131 temperature was kept at 22~27 °C, pH was 6.5~7.5, and dissolved oxygen was above 5.0 mg/L.

132 *2.2 Sample Collection and Preparation*

133 Three domesticated grass carp after six month of domestication were collected from ponds

134 (one fish each pond, with a body weight of 720 ± 50 g). Wild grass carp with a body weight of 550
135 ± 50 g were collected from same place where wild sub-adult grass carp were captured, and DGC
136 and WGC were at approximately the same age and ontogeny stage (i.e.,puberty), which was
137 determined by annulus characteristics of scale (Cai et al., 2020). Whole blood was collected from
138 were collected from the domesticated grass carp (sample DGC1 - DGC3) and wild grass carp
139 (sample WGC1 - WGC3) groups. Subsequently, blood samples were treated with EDTAK2 (Sanli,
140 Liuyang, China) and then centrifuged at 12,000 rpms for 3 min to separate red blood cells (RBCs)
141 from serum.

142 The experimental protocols used in this study were approved by the Laboratory Animal
143 Ethics Committee of Pearl River Fisheries Research Institute, CAFS, China, under permit number
144 LAEC-PRFRI-2021-06-03.

145 *2.3 Genomic DNA Extraction and Whole-Genome Bisulfite Sequencing*

146 Genomic DNA was extracted from RBCs of grass carp using DNeasy Blood and Tissue Kit
147 following the manufacturer' s protocol. Genomic DNA of RBCs from domesticated grass carp and
148 wild grass carp groups was sent to BGI (BGI Tech Solutions Co., Ltd., Shenzhen, China) for
149 whole-genome bisulfite sequencing. For normal WGBS library constructing, the DNA was
150 fragmented by sonication using a Bioruptor (Diagenode, Belgium) to a mean size of
151 approximately 250 bp, followed by the blunt-ending, dA addition to 3'-end, finally, adaptor
152 ligation(in this case of methylated adaptors to protect from bisulfite conversion), essentially
153 according to the manufacturer's instructions. Ligated DNA was bisulfite converted using the EZ
154 DNA Methylation-Gold kit (ZYMO). Different Insertsize fragments were excised from the same
155 lane of a 2% TAE agarose gel. Products were purified by using QIAquick Gel Extraction kit

156 (Qiagen) and amplified by PCR. At last, Sequencing was performed using the HighSeq4000 or
157 other Illumina platforms.

158 *2.4 Data Filtering and Reads Alignment*

159 After sequencing data was delivered, the raw reads were filtered by removing adaptor
160 sequences, contamination and low-quality reads from raw reads. Low-quality reads include two
161 types,and the reads meet any one of the two conditions will be removed: (1) Unknown bases are
162 more than10%; (2) The ratio of bases whose quality was less than 20was over 10%. After filtering,
163 the Clean data was then mapped to the reference genome of grass carp (Wang et al., 2015) by
164 BSMAP, and then remove the duplication reads and merge the mapping results according to each
165 library. The BSMAP script was BSMAP -a filename_1.clean.fq.gz -b filename_2.clean.fq.gz -o
166 filename.sam - d ref.fa -u -v 8 -z 33 -p4 -n 0 -w20 -s 16 -f 10 -L 100. The sam files were converted
167 to bam files using scripts (samtools view -S-b-o filename.bam filename.sam; samtools sort-m
168 2000000000 filename.bam filename.sort; samtools index filename.sort.bam). The mapping rate
169 and bisulfite conversion rate of each sample were calculated.

170 *2.5 Identification of Differentially Methylated Regions*

171 The methylation level was determined by dividing the number of reads covering each mC by
172 the total reads covering that cytosine (Lister R., 2009), which was also equal the mC/C ratio at
173 each reference cytosine (Xiang et al., 2010). The formula is Rmaverge = Nmall/Nmall+Nnmall.
174 Nm represents the reads number of mC, while Nnm represents the reads number of
175 non-methylation reads. Putative DMRs were identified by comparison of the sample DGC and
176 sample WGC methylomes using windows that contained at least 5 CpG (CHG or CHH) sites with
177 a 2- fold change in methylation level and Fisher test p value < = 0.05.

178 *2.6 Gene Ontology and Pathway Enrichment of DMRs*

179 GO enrichment analysis provides all GO terms that significantly enriched in a list of
180 differentially methylated genes, comparing to a genome background, and filter the differentially
181 methylated genes that correspond to specific biological functions.This method firstly maps all
182 differentially methylated genes to GO terms in the database (http://www.geneontology.org/),
183 calculating gene numbers for every term, then uses hypergeometric test to find significantly
184 enriched GO terms in the input list of differentially methylated genes, based on ' GO ::
185 TermFinder' (http://www.yeastgenome.org/help/analyze/go-term-finder). KEGG pathway
186 enrichment analysis helps to identify significantly enriched metabolic pathways or signal
187 transduction pathways in differentially methylated genes comparing with the whole genome
188 background. The calculating formula is the same as that in GO analysis.

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192

193 **3. Results**

194 *3.1 Global mapping and statistical analysis of the WGBS reads*

195 We conducted whole-genome bisulfite sequencing of grass carp blood from domesticated
196 grass carp group and wild grass carp group. After quality control of filtering, a total of 2.36 billion
197 clean reads were generated, consisting of 378.12 million, 387.63 million, and 321.7 million reads
198 for each domesticated grass carp sample and 371.48 million, 493.82 million, and 410.42 million
199 reads for each wild grass carp sample (Table 1). The bisulfite conversion rate (%) of all
200 sequencing libraries ranges from 99.16% to 99.28%. After read alignment, clean reads were
201 mapped to the reference genome of grass carp with mapping rates ranging from 88.03 % to

202 92.11 % (Table 1).

203

204 *3.2 Global DNA methylation patterns of domesticated and wild grass carp.*

205 The methylation levels of the whole genom were listed in Table 2. In each group,
206 approximately 9% of all genomic C sites were methylated. Methylation in grass carp was found to
207 exist in three sequence contexts: CG, CHG (where H is A, C, or T), and CHH. The average
208 methylation levels of CG, CHG, and CHH at the whole genome levels were 80.3%, 1.09%, and
209 1.08% in the domesticated grass carp group, and 75.04%, 1.07%, and 1.13% in the wild grass carp
210 group. The average methylation of CG showed a significantly increased within genome in the
211 domesticated grass carp group compared with the wild grass carp group ($p < 0.05$). The
212 methylation level of CHG and CHH showed no differences between domesticated grass carp and
213 wild grass carp groups ($p > 0.05$).

214 Methylation status of CG, CHG and CHH of grass carp genome showed the methylome' s
215 overall characteristics (Figure 1). The methylation levels of approximately 25% of all mCG were
216 hypermethylated (methylation level > 90%). However, only about 3% of mCHH and mCHG were
217 hypermethylated (methylation level > 90%) compared with mCG. The methylated Cs mostly
218 occur in the form of mCG, followed by mCHH and mCHG. The methylation level distribution of
219 mC and mCG were alike. The methylated Cs mostly occur in the form of mCG; approximately
220 96% of all detected mCs (Table 3). Proportion of mCHG range from 0.75% to 1.15%, and
221 proportion of mCHH range from 2.7% to 3.05% (Table 3). The proportion of mCG, mCHG and
222 mCHH showed no significant difference between domesticated grass carp group and wild grass
223 carp group ($p > 0.05$).

224 *3.3 DNA methylation levels of different genomic features*

225 Different genomic features show distinct methylation patterns which are related with distinct
226 regulation functions (Cokus et al, 2008). The heatmap presents the methylation landscape in
227 different genomic features (whole genome, CGI, downstream 2 kb, upstream 2 kb, mRNA, repeat,
228 CDS, exon), providing additional information as well as a global assessment of some of these
229 components (Figure2). CpG islands contained the highest numbers of CpG sites (approximately
230 10 - 20 CpG sites in a 200 bp window) compared with other genomic features. About 60% of CpG
231 sites in CpG islands were hypermethylated (methylation levels >90%) in the heatmap (Figure2
232 and Supplementary Material Figure S2). The other genomic features generally contained 0 - 10
233 CpG sites in the 200 bp window. A lower proportion of CpG sites within whole genome, mRNA
234 and repeat were hypomethylated (methylation levels < 10%) than CpG sites within remaining
235 genomic features (CGI, downstream 2 kb, upstream 2 kb, CDS, exon).

236 *3.4 DNA Methylation Patterns Across the Entire Transcriptional Units at Whole Genome Level*

237 In order to reveal the relationship between DNA methylation profiles and genes expression in
238 detail, Canonical DNA methylation profiles of the entire transcriptional units were divided into
239 distinct functional elements (Li et al., 2010) to study the changes of methylation levels in different
240 features (Figure 3). Methylation differences between CG and non-CpG methylation (CHG and
241 CHH) are visible (Figure 3), as methylation levels of CG are higher than those of CHG and CHH
242 across the entire transcriptional units. Another feature is a modest elevation in methylation level at
243 internal exons and internal intron during transcriptional unit scanning. The lowest methylation
244 level occurs in the first intron, followed by the first exons and down stream.

245 *3.5 Identification and Enrichment Analysis of Differential Methylated Regions*

246 To characterize the differences of genome methylation levels between domesticated grass

247 carp and wild grass carp groups, DMRs and differentially methylated genes (DMGs) were
248 detected. For CG context methylation, a total of 533,235 DMRs were identified between the two
249 groups, which corresponded to 20,010 DMGs in promoter regions and 27,016 DMGs in gene
250 body.

251 GO enrichment analysis of DMGs was performed to provide significantly enriched GO
252 terms corresponding to specific biological process, cellular component, and molecular function
253 (Figure 4). In promoter regions, the over-represented GO terms in the biological process are
254 cellular process, metabolic process, and biological regulation. The top enriched GO terms in the
255 cellular component are cellular anatomical entity, intracellular and protein−containing complex. In
256 terms of molecular function, the top enriched GO terms are binding, catalytic activity, and
257 molecular transducer activity. In gene body region, the over-represented GO terms in the
258 biological process are cellular process, metabolic process and biological regulation. The top
259 enriched GO terms in the cellular component are cellular anatomical entity, intracellular and
260 protein-containing complex. In terms of molecular function, the top enriched GO terms are
261 binding, catalytic activity and transporter activity. KEGG pathway analysis, which is an
262 alternative approach to categorize gene functions, was also conducted for the DMGs in promoter
263 and gene body regions. In promoter region, DMGs were significantly enriched in pathways in
264 cancer, neuroactive ligand−receptor interaction and PI3K−Akt signaling pathway. In gene body
265 region, DMGs were significantly enriched in metabolic pathways, pathways in cancer and MAPK
266 signaling pathway (Figure 4).

267 *3.6 Candidate DMGs associated with selection signatures.*

268 Many transcriptome and association studies have explored the molecular mechanisms

269 underlying selection signatures in fish and other species, laying a foundation for our investigation
270 of the involvement of DMGs in selection signatures. Beside, it is reported that DNA methylation,
271 especially in the promoter regions, usually affects gene expression by different modes (Moore et al,
272 2012). 38 candidate DMGs in promoter region were identified according to the following criteria:
273 (1) genes were differentially methylated in grass carp and wild grass carp groups; (2) genes were
274 enriched in pathways related to selection signatures; (3) genes were differentially expressed in fish
275 and other species or related with selection traits reported by previous studies. However, KEGG
276 pathway analysis showed that these methylated genes were not significantly enriched in predicted
277 process and signaling pathway (Figure 5). The protein-protein interaction network analysis
278 indicated that these DMGs were highly correlated with each other (Figure 6).

279 **4. Discussion**

280 *4.1 DNA methylation profiles in domesticated and wild grass carp*

281 Domestication is a process by which humans select some phenotypes of wild animal
282 species (i.e., morphological traits or growth). During the domestication process, some phenotypic
283 traits of animal could be altered by the artificial selection to help domesticated animal species
284 adapt to new environmental conditions (Sylvain et al, 2021). DNA methylation, one of the most
285 important and stable epigenetic modifications in eukaryotes, can lead to heritable phenotypic and
286 transcriptomic changes (Höglund et al., 2020). However, in aquatic species, there is still limited
287 research that illustrate the effects of DNA methylation on selection signatures by comparing
288 genome-wide methylation profiles between domesticated and wild fish species. In this study, we
289 identified some key differentially methylated genes related to selection signatures (including
290 growth and metabolism, immunity, foraging behaviors, social behaviors) in domesticated and wild

291 grass carp to uncover its genetic characters.

292 In the present study, we reported a genome-wide examination of DNA methylation in
293 domesticated grass carp and wild grass carp. The mapping rates of clean reads ranged from
294 86.25% to 92.11% (Table 1), which is above the average value reported in other fish species (Pan
295 et al., 2021). However, the WGBS results in this study are still consistent because bisulfite
296 conversion reduces the complexity of the genomic sequence and the ability of most computational
297 programs to align sequences onto the reference genome (Yang et al., 2020).

298 Further DNA methylation profile analysis was also conducted in our present study across the
299 distinct genomic features and entire transcriptional units. Our results showed that the
300 genome-wide methylation patterns were similar between two groups. Approximately 9% of all
301 total cytosines and 80% of CG were methylated and only a small proportions of methylation at
302 non-CpG methylation (CHG and CHH) was observed within all regions, which is consistent with
303 previous study (Cai et al., 2020). The high level of DNA methylation in CG content was a specific
304 characteristic of animals. DNA methylation in CHH and CHG patterns is a major characteristic of
305 plant methylomes and largely absent in animal methylomes (Zemach et al., 2010).

306 A modest elevation in methylation level at internal exons and intron was observed during
307 transcriptional unit scanning. The lowest methylation level occurs in the first exons, followed by
308 the the first intron and last exon. These results was consistent with previous findings in grass carp
309 (Cai et al., 2020), suggesting that mutagenic effects appear to occur at the first exons and internal
310 exon and intron tend to be influenced by the regulatory impact of DNA methylation.

311 To better understand the functional classification of DMGs during domestication, GO
312 enrichment analysis was conducted to gain insight into the biological processes in which the

313 DMGs might be involved. The top enriched GO terms of DMRs identified in the present study
314 were cellular process, biological regulation and metabolic process, suggesting that DNA
315 methylation may play a critical role in regulating global transcription during domestication.
316 Furthermore, to further explored the molecular mechanisms underlying selection signatures
317 in grass carp, 38 candidate DMGs in promoter region were identified, including IGF-1
318 (Insulin-like growth factor 1), GK (Glycerol kinase), GYS1 (Glycogen synthase 1), etc. But these
319 methylated genes were not significantly enriched in predicted process and signaling pathway of
320 KEGG.
321
322 *4.2 Key differentially methylated genes related to growth and metabolism*
323 Domestication is a long process which forces animals to adapt to captivity by modifying an
324 animal' s growth and metabolism, immune response, foraging and social behaviors. In the
325 process of domestication, fish undergo great change in the growth and metabolism due to the
326 difference of environmental conditions and food resource (Teletchea et al., 2016), which is closely
327 associated with the differential expression of genes involved in growth and metabolism (Shen et
328 al., 2021). In this study, several genes involved in growth and metabolism, such as IGF-1
329 (Insulin-like growth factor 1), GK (Glycerol kinase), GYS1 (Glycogen synthase 1), FASN (Fatty
330 acid synthase), etc, exhibited hypomethylation in domesticated grass carp group compared to wild
331 grass carp group. This result indicated that the expression of these genes might be upregulated. In
332 contrast, some genes related to growth and metabolism exhibited hypermethylation in
333 domesticated grass carp group, including G6PC (Glucose-6-phosphatase),
334 PCK1(Phosphoenolpyruvate carboxykinase), FBP1 (Fructose 1,6 bisphosphatase 1),
335 FOXO1(Forkhead box protein O1), ACAA1 (Acetyl-Coenzyme A acyltransferase 1), CPT1

336 (Carnitine palmitoyltransferase I), suggesting the expression of these genes might be
337 downregulated.

338 IGF-1, an important growth hormone, mediates the anabolic and linear growth promoting
339 effect of pituitary GH protein and has been well demonstrated to play an important role in the
340 skeletal muscle development (Laron et al., 2001). Thus, IGF-1 is thought to be a candidate gene
341 for regulating muscle growth.

342 GK is a key enzyme that catalyzes the first step in glycolysis (Enes et al., 2009). FOXO1,
343 G6PC, FBP1 and PCK1 are key enzymes of gluconeogenesis (Lu et al., 2018). Our results
344 indicated that, compared to wild grass carp, the glycolytic capacity is enhanced and the capacity
345 for gluconeogenesis is inhibited in domesticated grass carp feeding commercial diet. These results
346 was consistent with the previous finding that high-carbohydrate diet could induce glycolysis and
347 inhibit gluconeogenesis (Liu et al., 2021).

348 GSY1 is a rate-limiting enzyme in glycogen synthesis and plays an important role in the
349 synthesis of glycogen in the muscle (Martins et al., 2013). The present results suggested that the
350 capacity for muscle glycogen storage in domesticated grass carp maybe increase compared with
351 that of wild grass carp.

352 FASN, which catalyzes the last step in fatty acid biosynthesis, is believed to be the central
353 enzyme of hepatic lipid accumulation (Dorn et al, 2010). ACAA1, CPT1 gene play a certain role
354 in the lipid degradation (Song et al., 2019). In the present study, our results suggested that, in
355 domesticated grass carp, the ability to synthesize fat was enhanced and lipid degradation was
356 inhibited compared to wild grass carp, which may account for elevated lipid levels of muscle in
357 domesticated grass carp feeding commercial feed (Ashraf et al.,, 2011). According to the results,

358 an explanation for the enhanced fat deposition in fish might be excessive energy intake in
359 commercial diet (Wu et al., 2021). Overall, DNA methylation is likely to play an important role in
360 regulating growth and metabolism of domesticated grass carp by influencing these gene
361 expression.

362 *4.3 Key differentially methylated genes related to immunity.*

363 To maximize profitability, domesticated fish were cultured in intensive farming conditions
364 with limited space, high density and other stressors, which could influence fish immune response
365 negatively and even result in large-scale disease (Lin et al., 2018, Chen et al.,2017). It was
366 reported that, in the process of domestication, the immune status of domesticated fish could be
367 affected negatively due to increased chronic stress by confinement (Mandiki et al., 2011), which
368 would make fish more susceptible to pathogens and ultimately impair fish survival.

369 In this study, several immune related genes exhibited hypermethylation in domesticated grass
370 carp group compared to wild grass carp group, including MHCI (Major histocompatibility
371 complex class I), MHCII (Major histocompatibility complex class II), C1QA (Complement C1q
372 subcomponent subunit A), C3 (Complement C3), C4A (Complement C4-A), C5 (Complement C5),
373 IFN-γ (Interferon gamma), etc, which suggested the expression of these genes might be
374 downregulated. MHCI and MHCII are two cell surface proteins essential for the acquired immune
375 system for antigen presentation to recognize foreign molecules in vertebrates and can promote the
376 development and expansion of T cells (Huang et al., 2005). The complement system, an essential
377 part of both innate and adaptive immunity in teleosts, is initiated by one or a combination of three
378 pathways, the alternative, lectin and classical (Yang et al., 2016). IFN-γ, one of critical antiviral
379 cytokines, modulate functions of the immune system by up-regulate major histocompatibility

380 complex molecules (MHC I and MHC II) and directly activate other immune cells, such as
381 macrophages and natural killer cells (Borden et al., 2007). In our results, these immune related
382 genes mentioned above identified in domesticated grass carp exhibited hypermethylation,
383 indicating a poor immune performance in domesticated domesticated grass carp during long-term
384 domestication.

385 *4.4 Key differentially methylated genes related to foraging behaviors*

386 During the domestication process, fish change their foraging habits to obtain food in captive
387 conditions (Pasquet et al., 2018). In this study, foraging behaviors-related genes were also found to
388 be under strong selection in domesticated carp. The genes of GRM1(Metabotropic glutamate
389 receptor 1), TAS1R1 (Taste receptor type 1 member 1) and TAS1R3 (Taste receptor type 1
390 member 3) exhibited hypomethylation in domesticated grass carp group compared to wild grass
391 carp group, suggesting the expression of these genes might be upregulated. The TAS1R1 and
392 TAS1R3 heterodimer receptor functions as an umami receptor, responding to L-amino acid
393 binding, especially L-glutamate (Nelson et al., 2001). GRM1, which is widely expressed
394 throughout the central nervous system and regulates synaptic signaling, is another L-glutamate
395 receptor (Gabriel et al., 2009). Generally, these taste receptors play an important roles in
396 perception of L-amino acids and feeding behavior (Cai et al., 2021). Thus, these genes with
397 hypomethylation are likely to be upregulated in domesticated carp, facilitating the adaptation of
398 domesticated grass carp to commercial feed in artificial rearing condition.

399

400 *4.5 Key differentially methylated genes related to learning behaviors*

401 Learning and memory could enable the organism to plastically respond to the changing
402 environment. Increasing research has investigated the learning (cognitive) and memory

403 characteristics of fish in the past few decades, including spatial cognition, learned recognition,
404 social learning, foraging activity, etc (Dou et al., 2018). In this study, through domestication,
405 social learning-related genes have also been under selection pressure. The gene POMC
406 (Pro-opiomelanocortin) exhibited hypermethylation. In contrast, the genes of C-FOS
407 (Proto-oncogene C-FOS) and CBP (CREB-binding protein) exhibited hypomethylation in
408 domesticated grass carp group compared to wild grass carp group, suggesting the expression of
409 these genes might be upregulated. C-FOS is necessary for consolidation of non-spatial
410 hippocampal-dependent memory (Countryman et al., 2005), and C-FOS mRNA expressions are
411 up-regulated in response to a variety of neuronal activation protocols, including behavioral
412 training (Smulders et al., 2000) and long-term protentiation (Alberini et al., 2009). CBP is a
413 coactivator of transcription that play an essential role in memory consolidation (Korzus et al.,
414 2004). It was reported that gene expression of C-FOS and CBP facilitated the acquisition of novel
415 feeding habits in fish through the memory formation (Peng et al., 2019; Dou et al., 2018). Beside,
416 the C-FOS gene might be an important transcriptional factor to inhibit the expression of the
417 anorexigenic gene POMC, resulting in an increase of the food intake of dead prey fish in mandarin
418 fish (Peng et al., 2019). Therefore, it tempts us to speculate that the learning-related genes of
419 C-FOS and CBP may be responsible for the acquisition of novel feeding habits in domesticated
420 grass carp, which considerably increase the rate of domestication. Furthermore, individual food
421 intake is enhanced through the interaction between the learning gene C-FOS and the appetite
422 control gene POMC, contributing to the faster growth of domesticated grass carp indirectly.

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425

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580 **Table 1**

581 Alignment statistics with reference genome

Sample ID	Clean Reads	Mapped Reads	Mapping Rate (%)	Uniquely Mapped Reads	Uniquely Mapping Rate (%)	Bisulfite Conversion Rate (%)
DGC1	378,121,760	347,944,087	92.02	329,583,800	87.16	99.28
DGC2	387,627,636	353,434,830	91.18	335,393,994	86.52	99.23
DGC3	321,704,534	296,306,210	92.11	281,433,956	87.48	99.16
WGC1	371,478,196	321,457,393	86.53	300,678,599	80.94	99.16
WGC2	493,819,092	425,920,306	86.25	398,434,532	80.68	99.24
WGC3	410,419,590	361,301,972	88.03	339,621,985	82.75	99.22

582 Note: DGC, domesticated grass carp; WGC, wild grass carp

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595 **Table 2**

596 Average methylation levels of different genomic regions.

Groups	Regions	C (%)	CG (%)	CHG (%)	CHH (%)
DGC1	Genome	9.76	80.38	0.99	1.01
DGC2	Genome	9.69	80.55	1.18	1.06
DGC3	Genome	9.68	79.98	1.11	1.17
WGC1	Genome	9.21	74.7	1.12	1.18
WGC2	Genome	9.19	75.04	1.02	1.09
WGC3	Genome	9.13	75.39	1.07	1.12

597 Note: DGC, domesticated grass carp; WGC, wild grass carp. CG, CHG, and CHH

598 (where H is A, C, or T)

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603 **Table 3**

604 Proportion of CG, CHG and CHH in all Methyl-cytosine

		mCG	mCHG	mCHH
DGC1	mC number	26,890,172	208,311	750,652
	proportion (%)	96.557	0.748	2.695
DGC2	mC number	26,953,558	323,600	784,048
	proportion (%)	96.053	1.153	2.794
DGC3	mC number	26,520,365	219,138	811,594
	proportion (%)	96.259	0.795	2.946
WGC1	mC number	26,700,829	228,299	848,145
	proportion (%)	96.125	0.822	3.053
WGC2	mC number	27,258,571	220,189	831,299
	proportion (%)	96.286	0.778	2.936
WGC3	mC number	26,934,911	222,172	821,714
	proportion (%)	96.269	0.794	2.937

605 Note: DGC, domesticated grass carp; WGC, wild grass carp. CG, CHG, and CHH

606 (where H is A, C, or T)

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Figure 1. Distribution of methylation level of mC in each sequence context .

The x-axis was defined as the percentage of reads showing mC at a reference cytosine site. The y-axis indicates the fraction of total mC calculated within bins of 10%. DGC, domesticated grass carp; WGC, wild grass carp. (A) Sample DGC 1; (B) Sample DGC2; (C) Sample DGC3; (D) Sample WGC1; (E) Sample WGC2; (F) Sample WGC3.

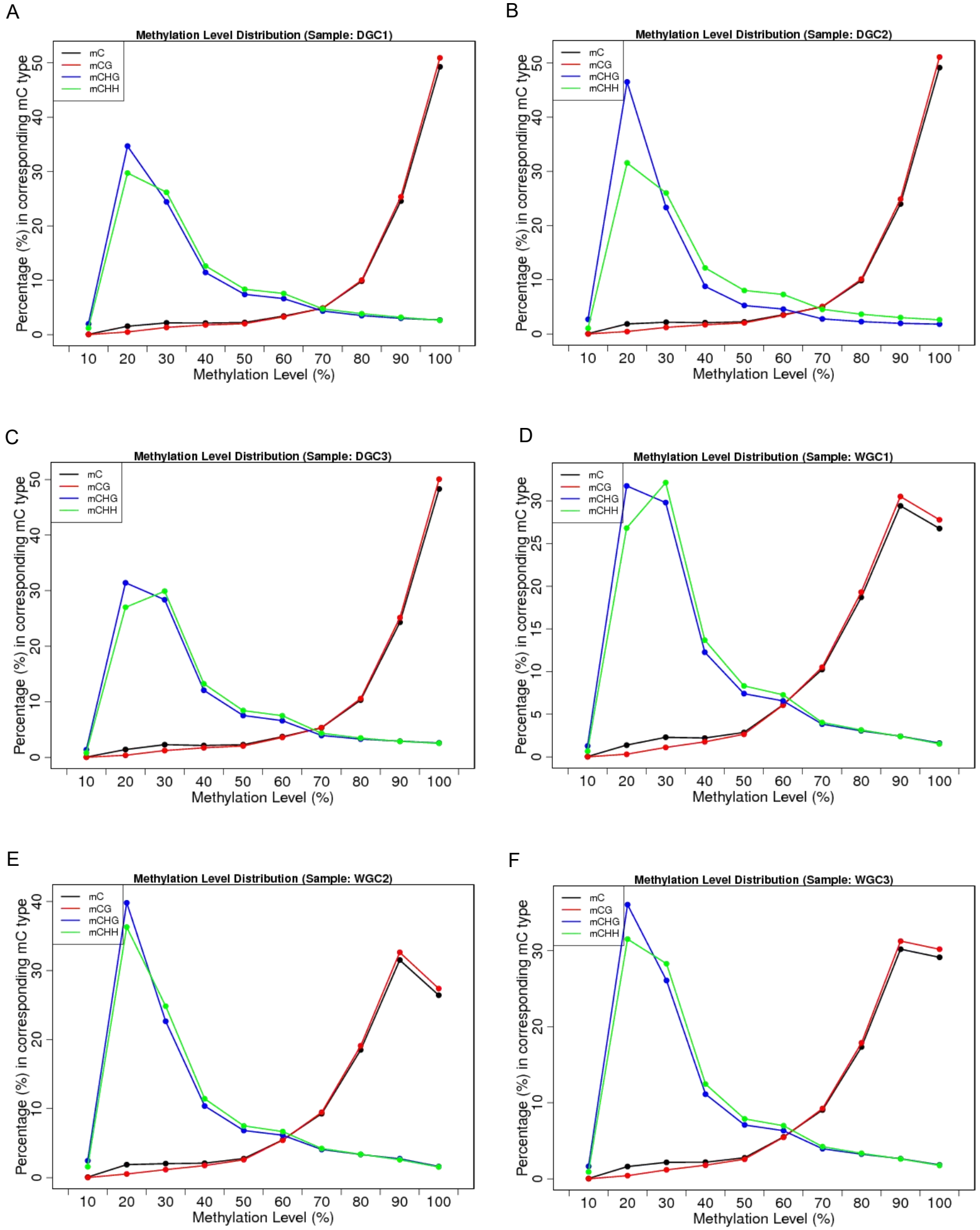
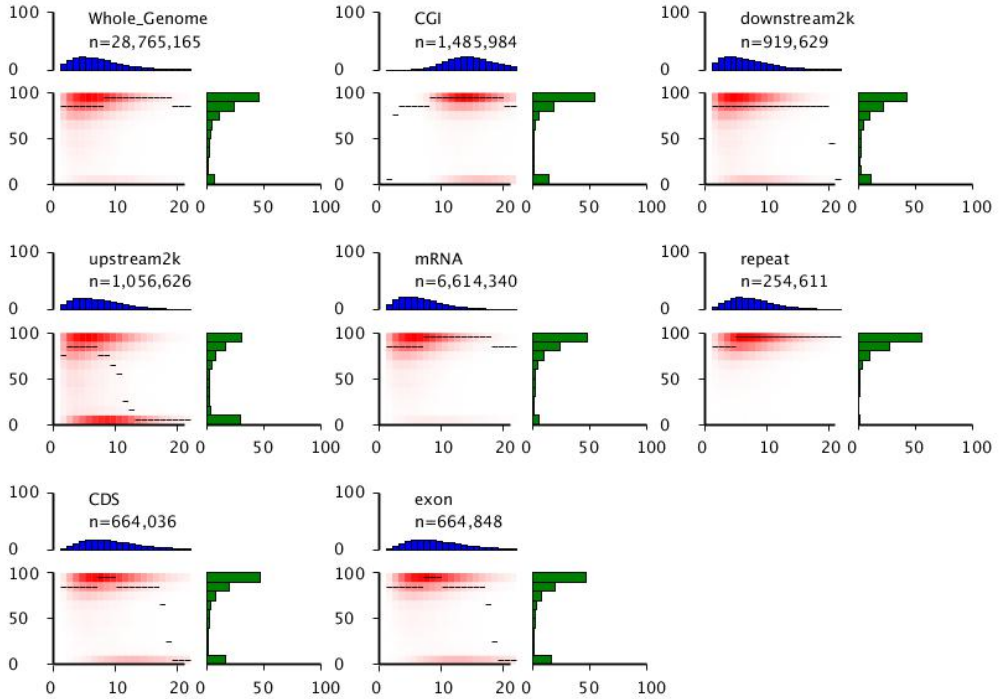


Figure 2. Heat maps show distinct methylation and CpG density patterns.

CpG density (x-axis) is defined as numbers of CpG dinucleotides in 200bp windows. Methylation level (y- axis) is defined as average methylation level of cytosines in CpGs. The thin black lines within each heat map denote the median methylation level of CpGs at the given local density. The red color gradient indicates abundance of CpGs that fall into bins of given methylation levels and CpG densities. The blue bar charts above each heat map show the distribution of CpG densities, projected onto the x-axis of the heat maps. The green bar charts to the right of the heat maps show the distribution of methylation levels, projected onto the y-axis of the heat maps. DGC, domesticated grass carp; WGC, wild grass carp. (A) Sample DGC 1; (B) Sample DGC2; (C) Sample DGC3; (D) Sample WGC1; (E) Sample WGC2; (F) Sample WGC3.

A



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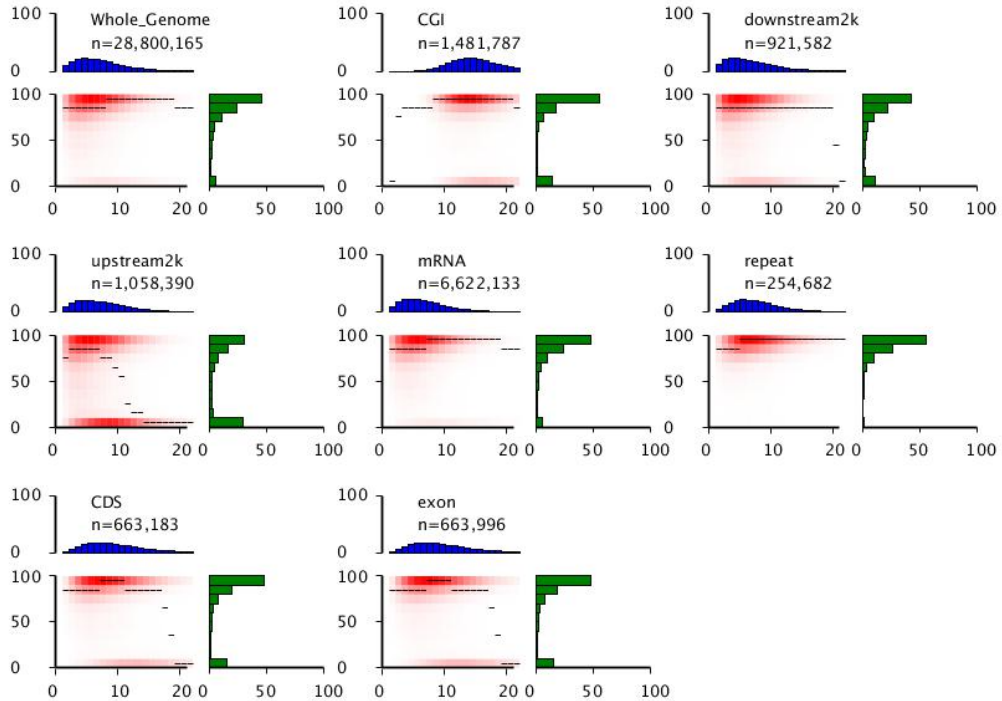
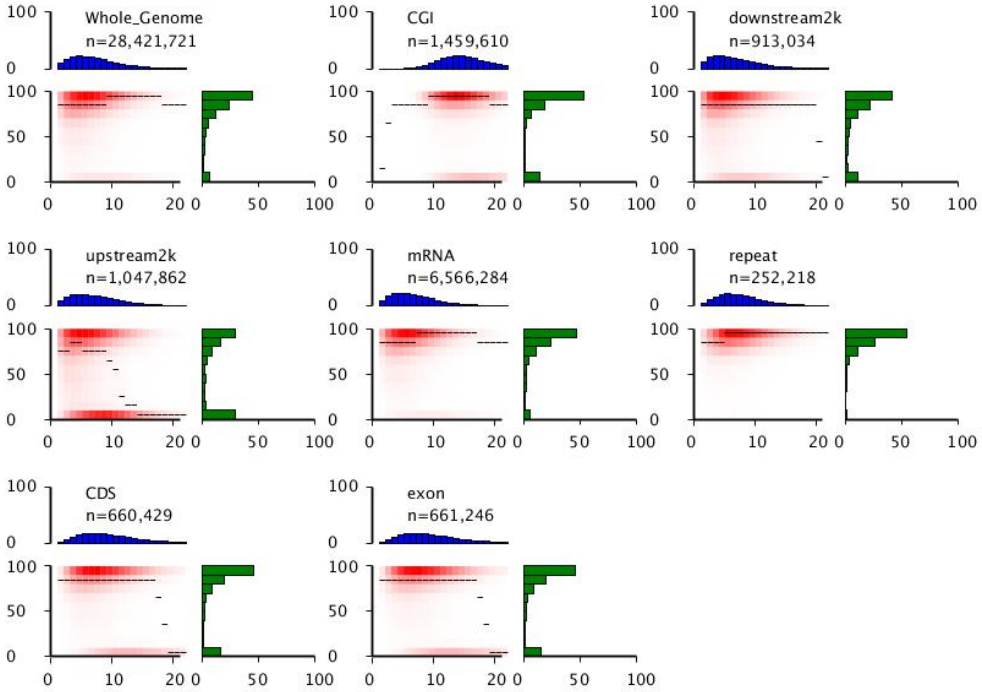


C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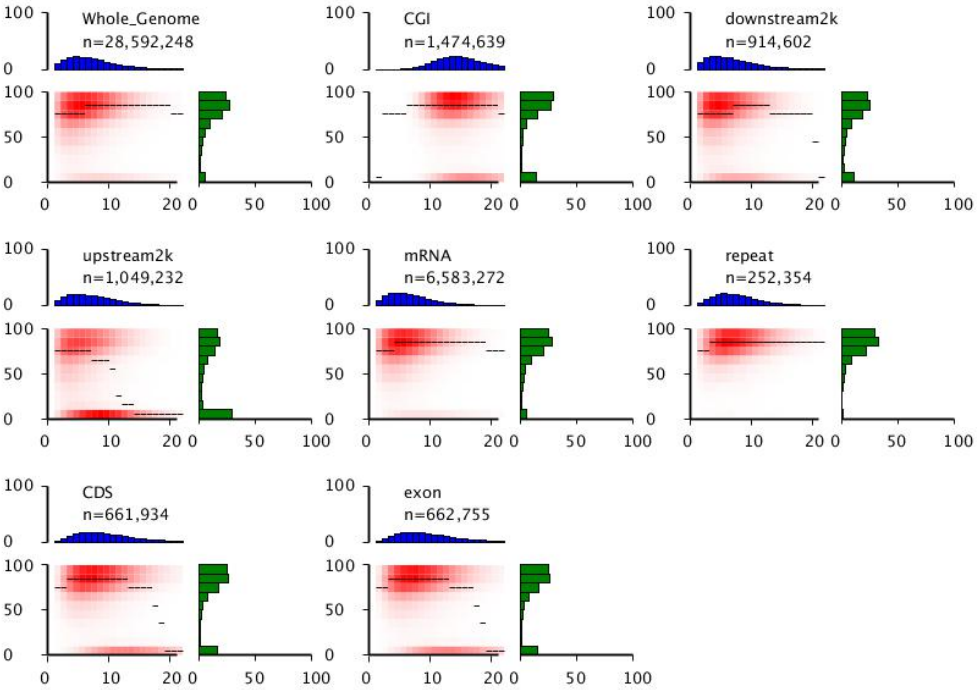
CGI
n=1,459,610
downstream2k
n=913,034
upstream2k
n=1,047,862
mRNA
n=6,566,284
repeat
n=252,218
CDS
n=660,429
exon
n=661,246

D


Whole_Genome
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CGI
n=1,474,639
downstream2k
n=914,602
upstream2k
n=1,049,232
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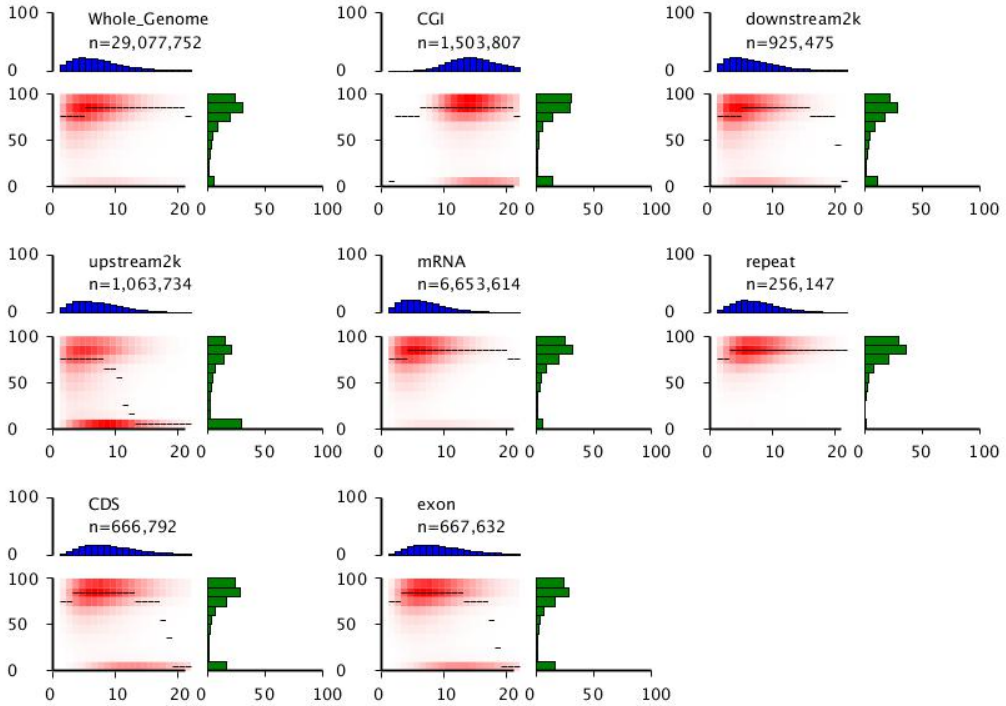
n=6,583,272
repeat
n=252,354
CDS
n=661,934
exon
n=662,755

E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upstream2k
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exon
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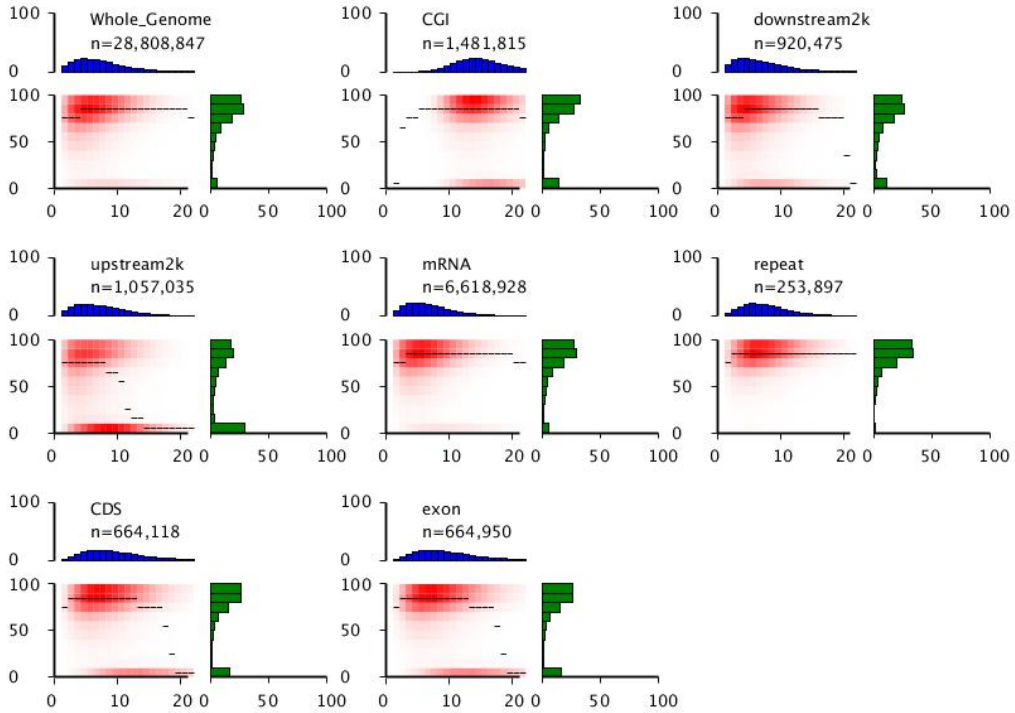
F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upstream2k
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mRNA
n=6,618,928
repeat
n=253,897
CDS
n=664,118
exon
n=664,950

Figure 3. DNA Methylation Patterns Across the Entire Transcriptional Units at Whole Genome Level of sample DGC1. The canonical gene structure is defined by 7 different features, denoted by the x-axis. The length of each feature was normalized and divided into equal numbers of bins. Each dot denotes the mean methylation level per bin and the respective lines denote the 5-bin moving average. Each feature was analyzed separately for the numbers listed in the table below the figure. The green vertical line indicates the mean location of the transcription start sites. DGC, domesticated grass carp; WGC, wild grass carp. (A) Sample DGC 1; (B) Sample DGC2; (C) Sample DGC3; (D) Sample WGC1; (E) Sample WGC2; (F) Sample WGC3.

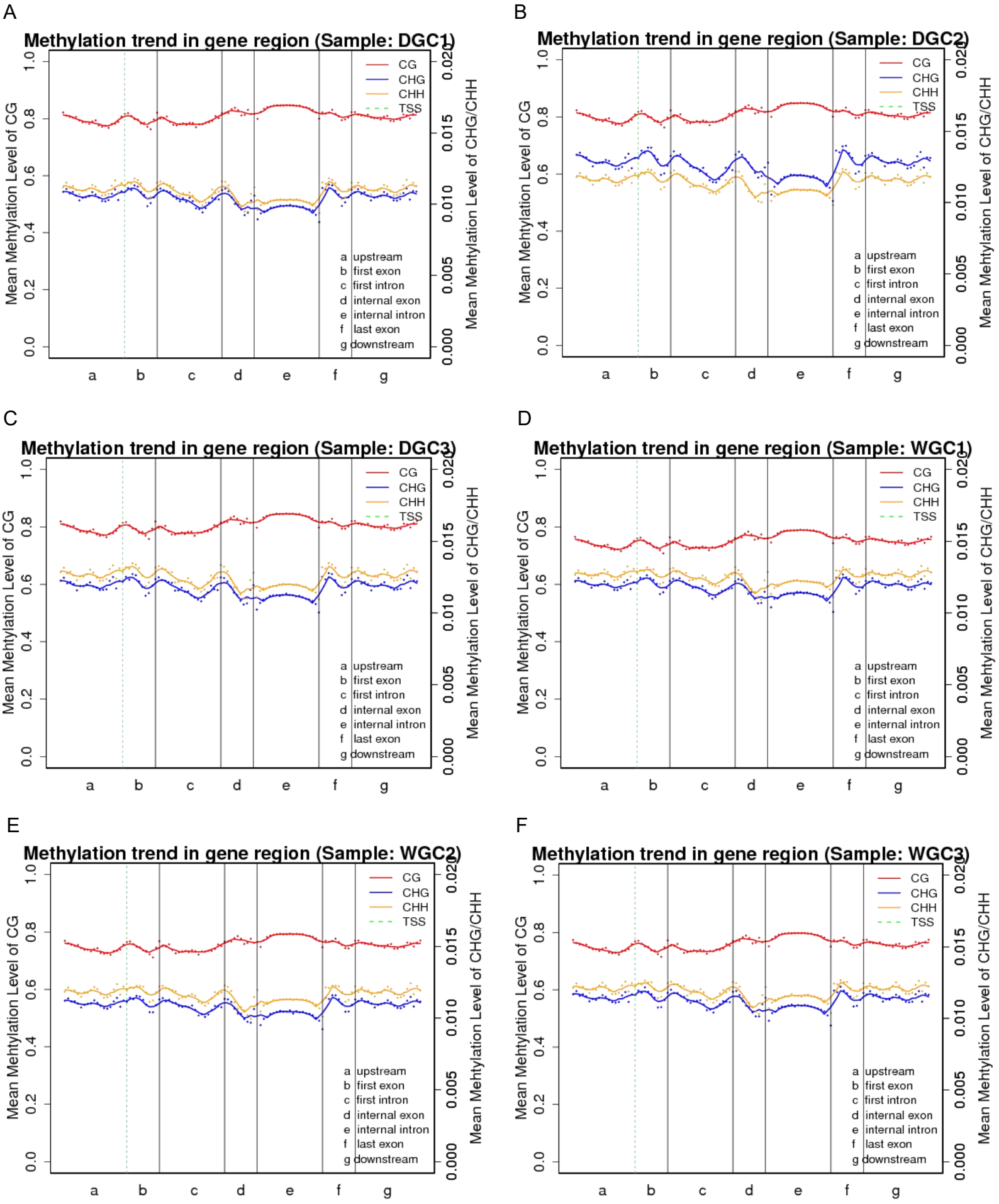
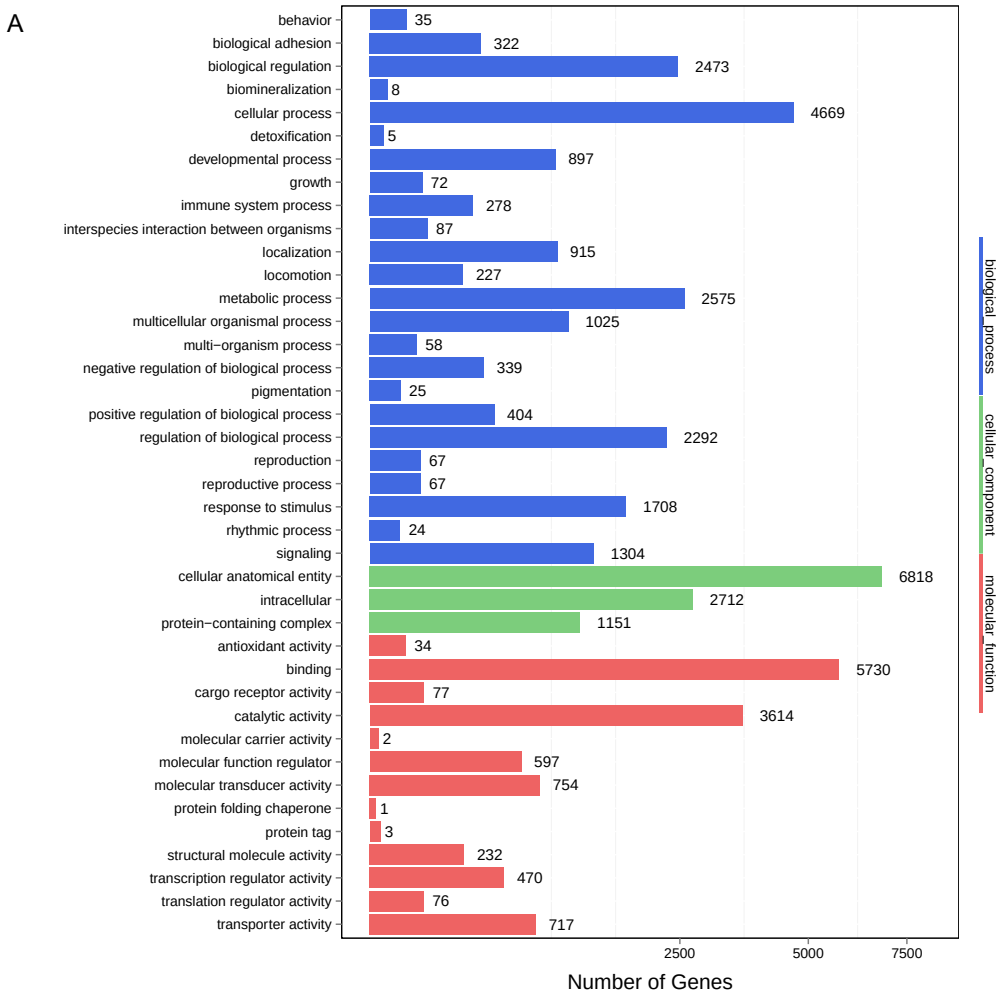


Figure 4. GO analysis of differentially methylated regions (DMRs)-related genes.

The x-axis represents three domains of GO while the y-axis represents the gene number in every pathway and processes.

(A).GO analysis of differentially methylated regions (DMRs)-related genes in promoter region

(B).GO analysis of differentially methylated regions (DMRs)-related genes in gene body region



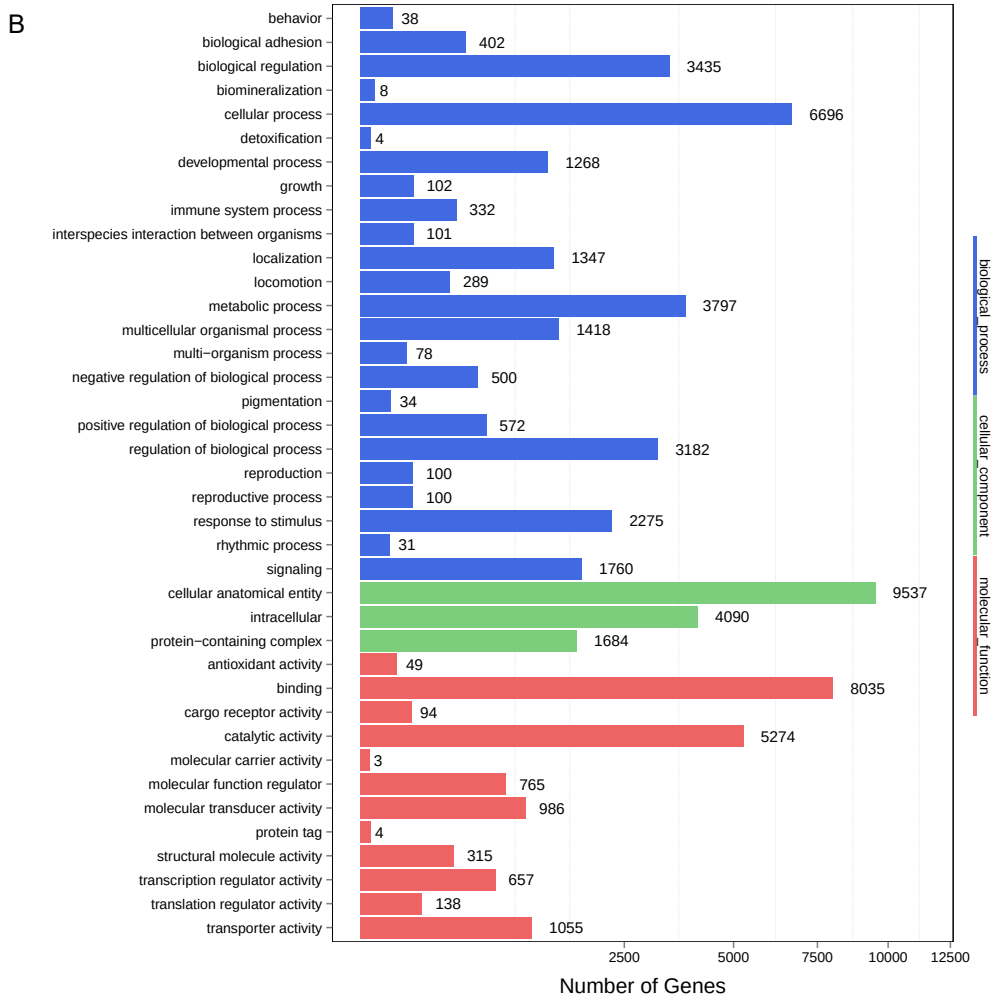
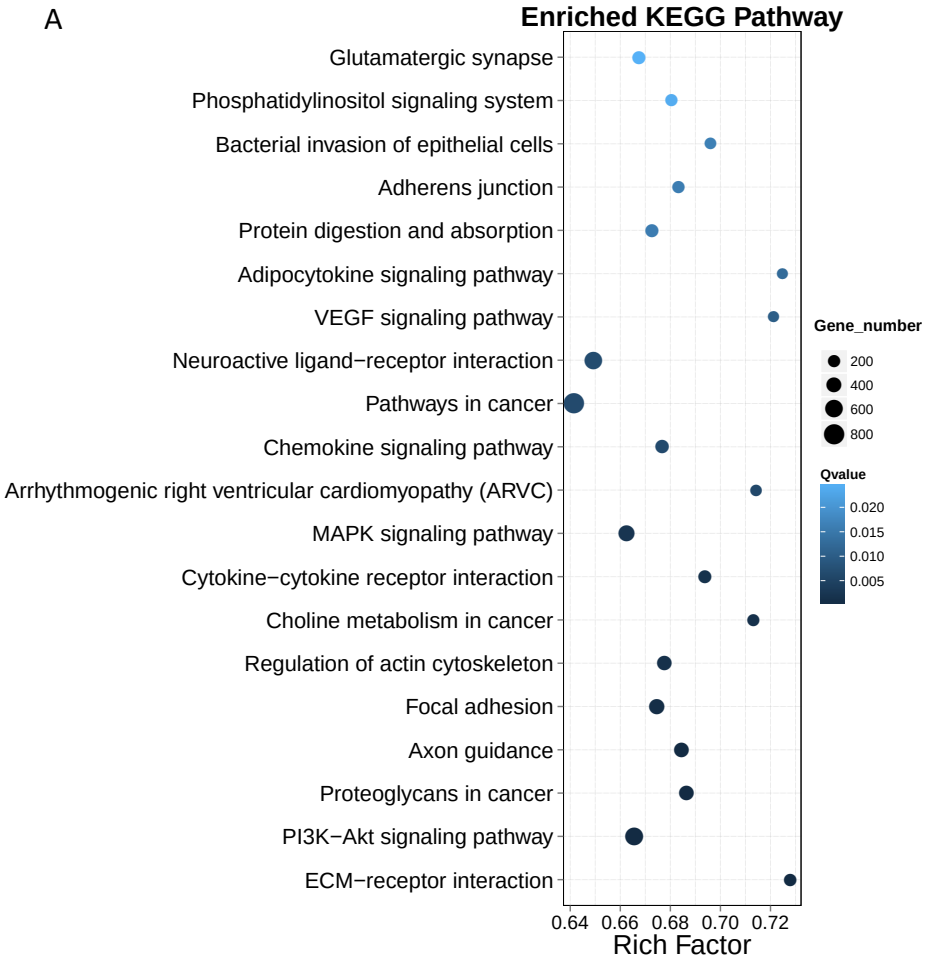


Figure 5. Pathway analysis of DMRs-related genes

The abscissa represent the richness factor, the ordinate represent the enriched pathway terms. Q-value represents the corrected P, and a small Q-value indicates high significance.

(A)Pathway analysis of DMRs-related genes in promoter region; (B) Pathway analysis of DMRs-related genes in gene body region.



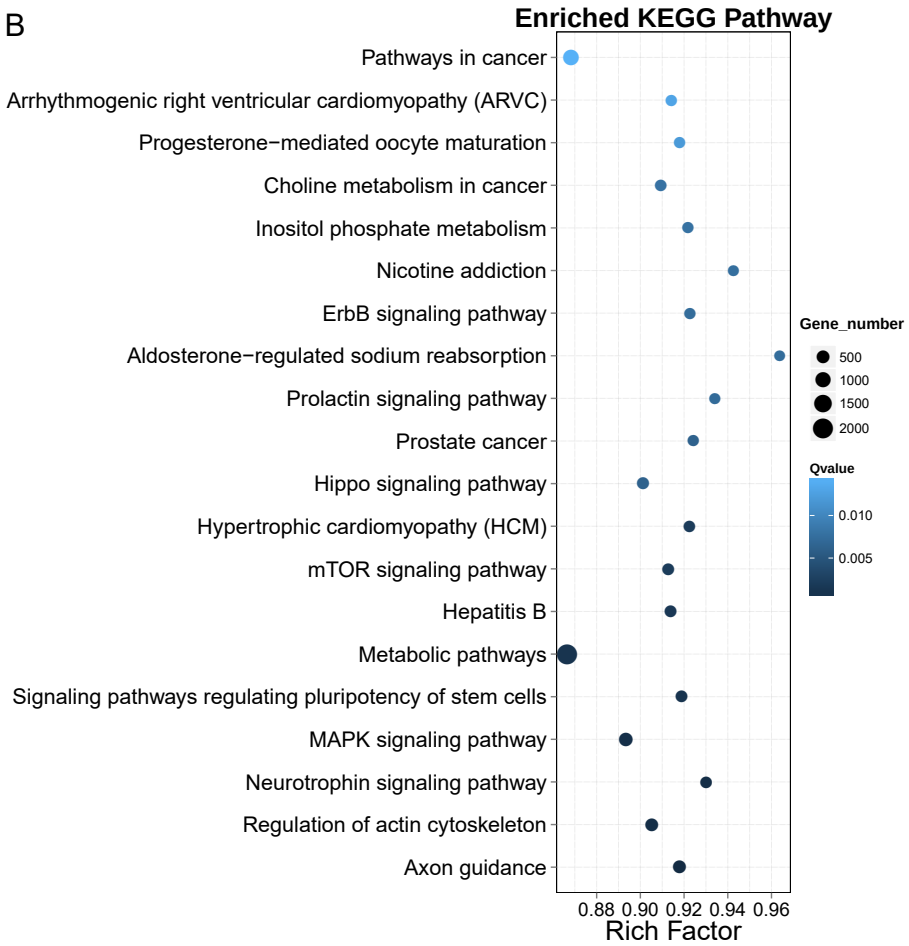
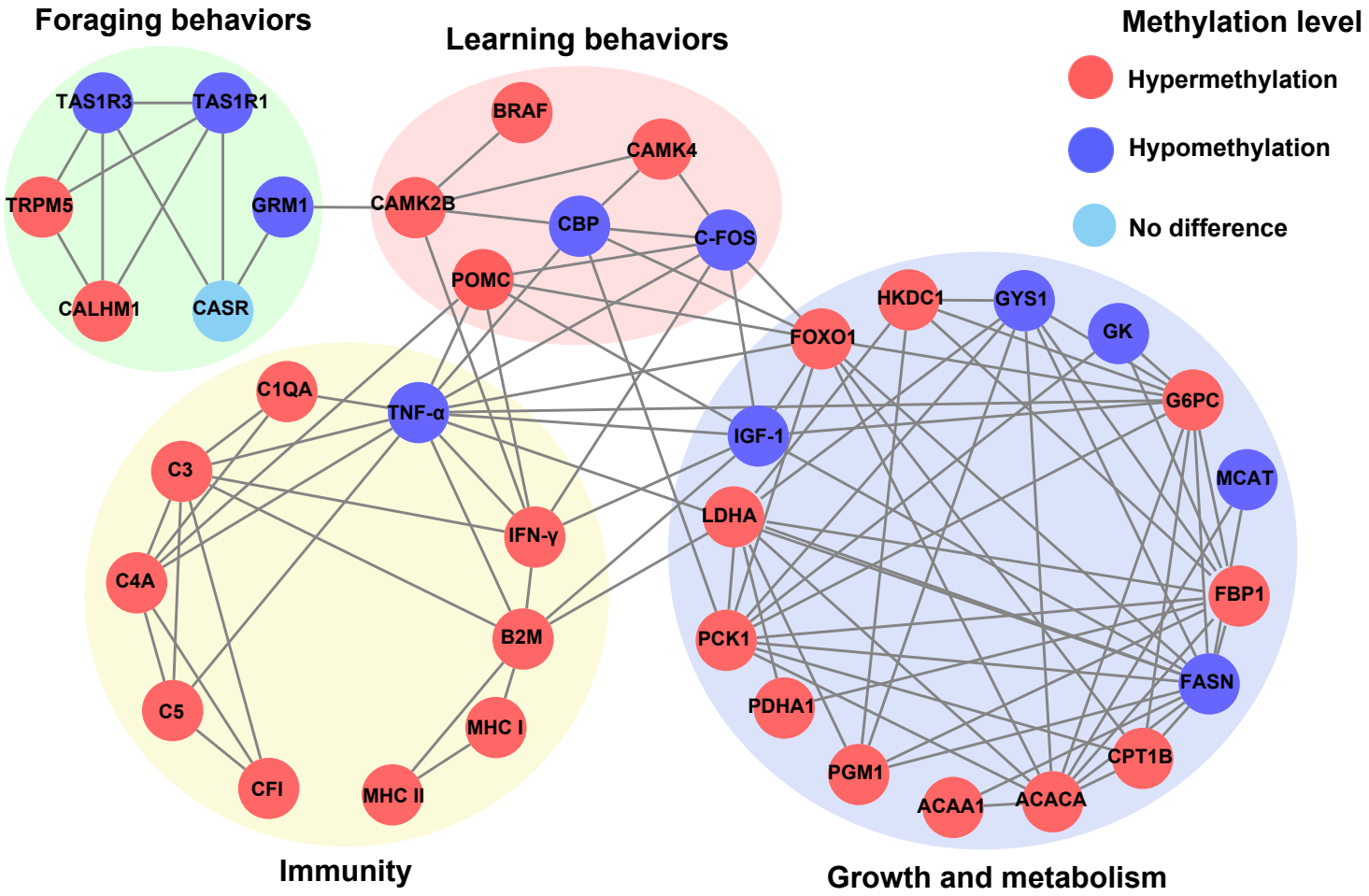



Figure 6. Protein-protein interaction network analysis of DMGs in different selection signatures.