Chloroplast DNA methylation in the kelp *Saccharina latissima* is determined by origin and influenced by cultivation

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Abstract

Chloroplast DNA is methylated in the kelp *Saccharina japonica*, in contrast to most plants. Its function is yet largely unexplored. We detected methylation in the chloroplast DNA of the congener *Saccharina latissima*, a non-model macroalgal species of high ecological (wild populations) and economical (wild and cultured populations) importance in the North Atlantic. To the functional relevance of chloroplast DNA methylation, we compared for the first time methylation patterns between wild and cultured kelp from different climatic regions (High-Arctic (79 °N) and temperate (53 °N), laboratory samples at 5 °C, 10 °C and 15 °C). Our results suggest genome-wide differences in methylated sites, and methylation level, between the climatic regions. At gene level, our data found functions related to photosynthesis to be the predominant affected case only for differential methylation between origins, but not between growth conditions. Here, sample origin led to significant differences between cultivated and wild samples due to differential methylation of genes related to DNA replication in the Spitsbergen samples. Both findings indicate that origin and cultivation

strongly affected the chloroplast methylome, but differently. Similar methylomes for samples from the same origin – independent from whether they grow in the wild or in the lab – suggest that origin-specific methylation marks on the chloroplast genome are inherited. However, the capacity for rapid adaptation (to cultivation conditions) could be shown for *Saccharina latissima* during this study. Given that DNA-methylation affects gene expression, our study suggests that lab-cultivation alters epigenetically determined kelp chloroplast characteristics at least to the same degree as ecotypic differentiation does.

Keywords:

Chloroplast, organelle genome methylation, epigenetics, non-model organism, cultivation, aquaculture, marine macrophyte, marine algae

Introduction

Primary production, by which photoautotrophic organisms convert oxidised inorganic carbon into organic carbon via photosynthesis, is the one crucial process that ensures the existence of nearly all food webs, both terrestrial and marine. Since chloroplasts are presumed to have developed from a free-living cyano-bacteria to an endosymbiontic organelle (Nishimura 2010), they feature their own, albeit reduced, genome. Due to the reduced nature of their genome, the organelles are semi-autonomous, and depend on extra-organellar products for their internal processes (Wang et al. 2020). Epigenetic mechanisms, including histone modification, non-coding RNA, and DNA methylation (Boquete et al. 2021), have been shown to play vital roles in many cellular processes, such as regulation of gene expression, mRNA processing, timing of DNA replication, determination of chromatin structure, or silencing of transposons (Finnegan et al. 1998; Zhang et al. 2018). DNA methylation is the most stable epigenetic mechanism that can persist for multiple generations (Lämke and Bäurle 2017), thus is presumed to be most relevant for epigenetic adaptation. In contrast, histone modifications

52 and ncRNAs are often only stable for several days or weeks (Lämke and Bäurle, 2017).
53 However, the function of chloroplast genome methylation is yet unclear, but intensely
54 researched in the few species of mostly agricultural use that show chloroplast methylation,
55 such as tomato, rice, or the unicellular green alga Chlamydomonas (Kobayashi et al. 1990;
56 Nishimura et al. 1999; Muniandy et al. 2019). In marine primary producers, the first
57 chloroplast genome methylation has recently been documented in the brown macroalga
58 *Saccharina japonica* (Teng et al. 2021). Its functional relevance remains unknown.

59 Macroalgae are the predominant source of coastal marine primary production (Field et al.
60 1998; Filbee-Dexter 2020). Nearly exclusively restricted to the shorelines of the continents,
61 macroalgae act as foundation species of marine rocky shore ecosystems, in addition to
62 forming the base of food webs, and hence, have high and diverse ecological value (Bartsch et
63 al. 2008; Teagle et al. 2017; Duffy et al. 2019). Their economic value is increasing exponentially
64 as macroalgae cultivation gains momentum outside of Asia as an environmentally sustainable
65 new blue economy with a multitude of applications (Cai et al. 2021). In 2019, marine
66 macroalgae accounted for ~30% of the global production of marine aquaculture commodities
67 (120 million t), worth US$ 14.7 billion (Cai et al. 2021). However, in recent years macroalgae
68 distributions have shifted northward in response to increasing (summer) water temperatures
69 (Krause-Jensen et al. 2020; Bringloe et al. 2020). Heat waves affect both wild populations and
70 aquaculture yields. In kelp, the respective thermal tolerance of gameto- and sporophyte
71 stages seems to be the strongest factor defining their global distribution limits (Monteiro et
72 al. 2019; Liesner et al. 2020). Temperature is a controlling factor in many biological processes
73 such as photosynthetic carbon acquisition. Enzymatic activity of RubisCO, the carbon
74 capturing enzyme in photosynthesis, increases by a factor of 7 between 5°C and 15°C (Cen and
75 Sage 2005), and has been shown to be especially vulnerable to high temperatures. As the

76 chloroplast genome carries several photosynthesis-relevant enzymes, investigating its
77 methylation at different temperatures is particularly relevant when considering methylome-
78 based temperature adaptation in accordance to the eco-genotype approach (Scheschonk et
79 al. 2022). While epigenetic mechanisms have been extensively researched in terrestrial plants,
80 knowledge about kelp epigenetics still is limited to a handful of studies (Cock et al. 2010; Fan
81 et al. 2020a; Scheschonk et al. 2022), with a single study on chloroplast DNA methylation (Teng
82 et al. 2021). In many primary producers, the chloroplast genome can be used as control to
83 estimate the rate of false positive methylation calls, as the chloroplast genome commonly has
84 been presumed to be un-methylated, despite data suggesting otherwise (*Saccharina japonica*,
85 Teng et al 2021; Chlamydomonas reinhardtii, Nishimura 2010; Nishimura et al. 2002;
86 Nishimura et al. 1999; Nishiyama et al. 2004; Nishiyama et al. 2002; Umen and Goodenough
87 2001). Epigenetic mechanisms hold the key to rapid local adaptation, and hence the capacity
88 for species survival and ecosystem stabilisation despite swift alterations in biotic and abiotic
89 factors. Especially in the face of the soaring changes in local and global ecosystems due to the
90 anthropogenic climate crisis, gaining knowledge in this field is crucial. Our study is the first
91 attempt to determine the influence of temperature, origin, and growth condition (wild *vs*
92 cultivated) on the methylome of chloroplasts of the kelp *Saccharina latissima* (sugar kelp). In
93 the northern hemisphere, this boreal-temperate marine brown alga, a congener species to *S.*
94 *japonica* with a large latitudinal distribution range, is among the macroalgal species of both
95 high ecological and economic value (Bartsch et al. 2008; Cai et al. 2021). In this fundamental
96 research study, we tested the following hypotheses:

97 1) *Saccharina latissima* features chloroplast genome methylations, as has been reported for
98 *S. japonica* (Teng et al 2021).

99 2) The chloroplast methylome mirrors the nuclear methylome (Scheschonk et al. 2022) in
100 showing strongest epigenetic differentiation between origins, with modifications specific to
101 temperature and lab cultivation.

102 Results

103 *Methylation levels and methylated sites*

104 According to *in-silico* digestion, there were 69 potential MethylRAD sites for CG methylation,
105 and 201 for CHG methylation in the chloroplast genome of *Saccharina latissima*. 20.7% and
106 24.8% of these sites were methylated, respectively (Table 1).

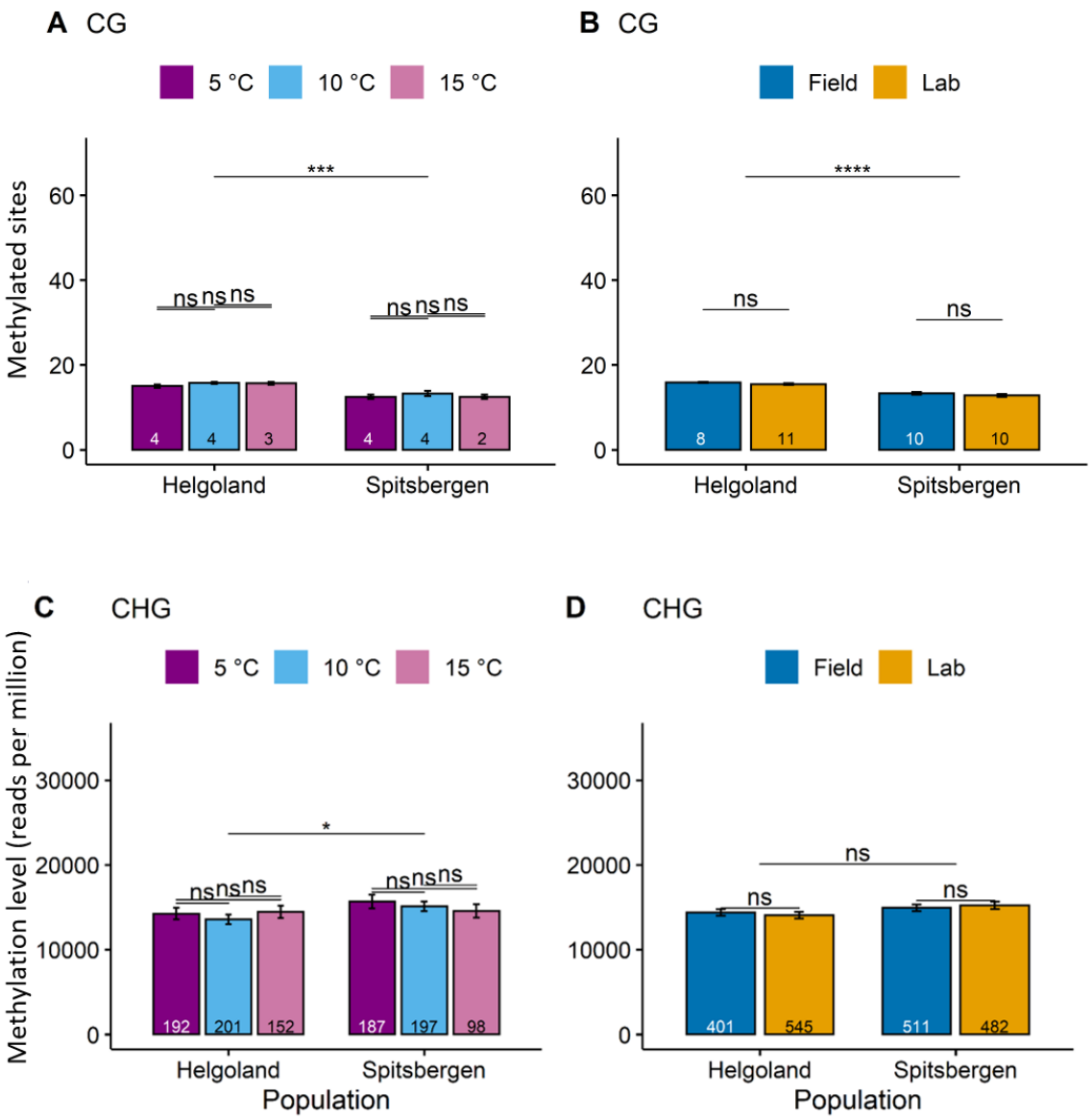
107 Table *1:* Potentially methylated sites for each sequence context, the mean number of methylated sites
108 per sample and standard deviations for the means. Methylation level is the mean percentage of
109 potential methylation sites methylated in samples, shown in the table with standard deviations (SD).

Sequence Context	Potential Sites (in-silico)	Methylated sites (mean +/- SD)	Methylation level (mean +/- SD)
CG	69	14.3 ± 1.5	20.7% ± 2.2%
CHG	201	49.8 ± 3.0	24.8% ± 1.5%

110

111 The number of methylated CG sites was significantly higher in Helgoland than in Spitsbergen
112 samples both in laboratory samples ($p < 0.001$, Fig. 1 A) and in laboratory and field samples
113 combined ($p < 0.0001$, Fig. 1 B), but no significant differences were found between treatment
114 temperatures or between field and laboratory conditions for sites in CHG contexts (Suppl. Fig.
115 1 C, D).

116 Methylation levels normalized to reads per million (RPM) showed no significant differences in
117 CG contexts between any groups (Suppl. Fig. 1 A, B), but the Spitsbergen population showed
118 higher methylation levels than the Helgoland population in the laboratory samples ($p < 0.05$,
119 Fig. 1 C). There were no significant differences between field and lab conditions in CHG context
120 (Fig. 1 D).



121 Figure *1*: **Significant differences in methylated sites and methylation level between conditions.**
122 Methylated CG sites (A, B), and methylation levels as reads per million (RPM) for CHG sites (C, D)
123 between populations and temperatures in laboratory samples (A, C) and compared between lab and
124 field samples (B, D). ns: not significant. Numbers at the bottom of the bars indicate sample size.
125 Significance codes after p-value correction (Benjamini-Hochberg) for multiple comparisons: '< 0.05': *,
126 '< 0.01': **, '< 0.001': ***, '< 0.0001': ****.

Principal Component Analysis

Laboratory and field samples formed distinct groups along the first dimension with 95% confidence ellipses not overlapping when both origins were combined (Fig. 2 A). The first two principal components equally contributed about 14 % of the total sample variation, which was similar between the comparisons of 'lab vs field' (Fig. 2 A) and 'Helgoland vs Spitsbergen' (Fig. 2 B). However, for the origins (Fig. 2 B), the populations of Helgoland and Spitsbergen formed distinct groups along the second dimension when lab and field samples were combined (Fig. 2 B), while when field and laboratory samples were separately analysed for origin, Helgoland and Spitsbergen samples again split along the first axes (Fig. 2 C, D).

Temperatures in the laboratory samples (5°C, 10°C, 15°C) did not separate into distinct groups (Fig. 2 E).

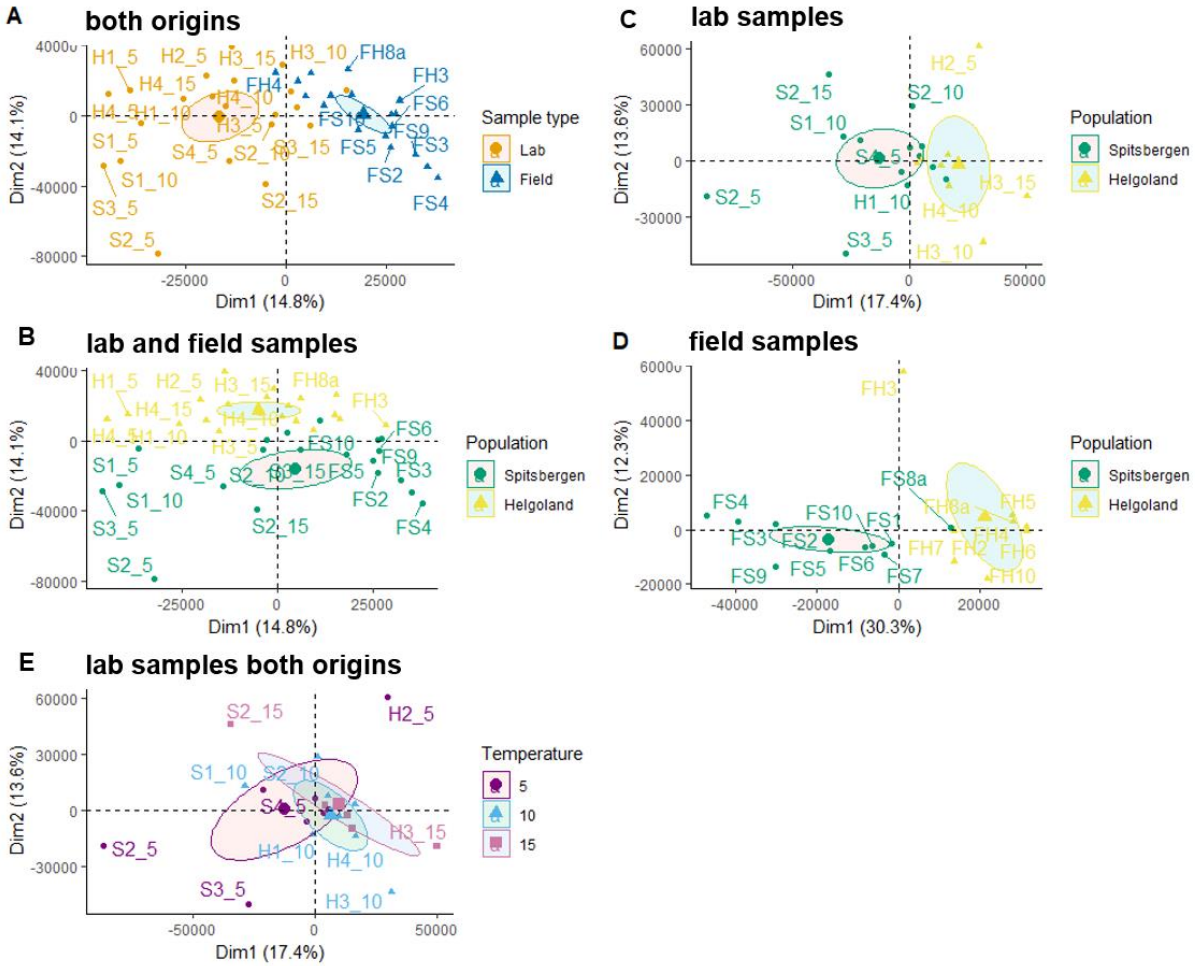


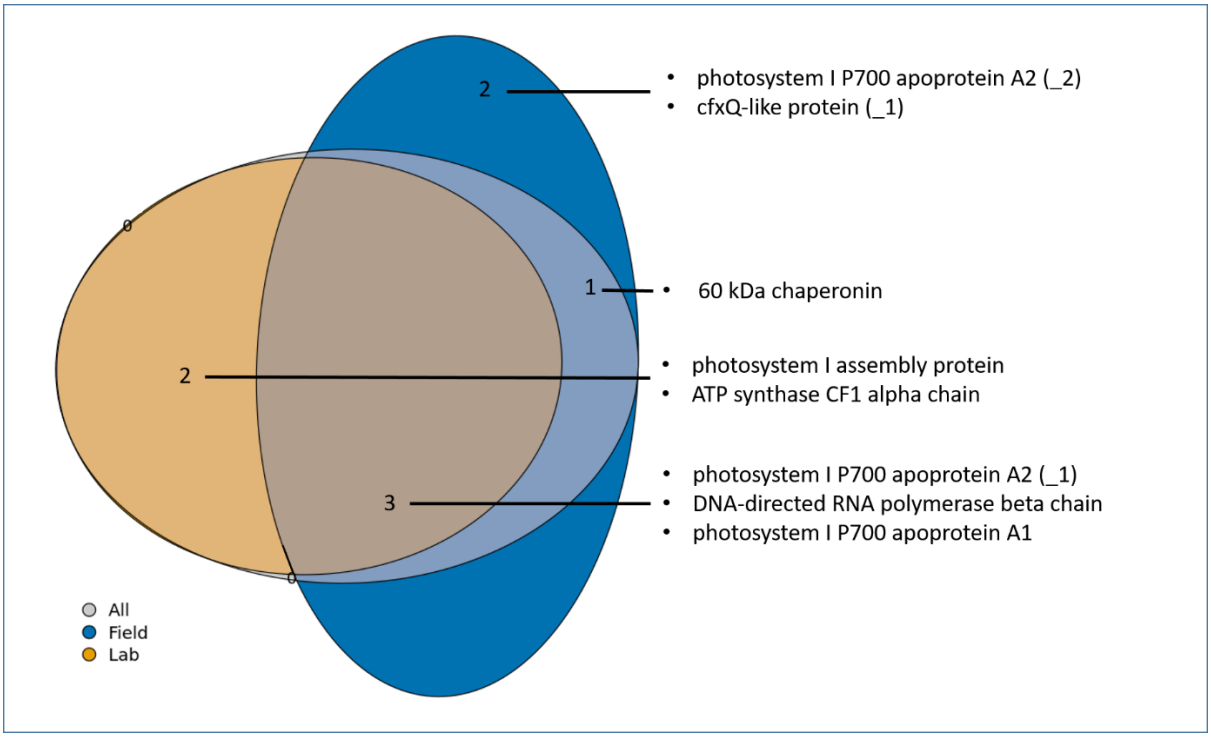
Figure *2*: **Principal Component Analysis for A: all samples, grouped by laboratory or field samples. B: laboratory samples grouped by origin**. C: all samples, grouped by origin. D: field samples grouped by origin. E: laboratory samples of both origins grouped by temperature treatment. Ellipses represent 95% confidence intervals around the group medians.

Differentially methylated sites and GO terms

Comparing the origins (Helgoland vs Spitsbergen, Fig. 3), differentially methylated sites were as often higher methylated in Spitsbergen samples as in Helgoland samples (Suppl.Tab.1-3). Of the eight genes with significant methylation differences between the origins (Fig. 3), only three genes (psaB_(1), atpA, yfc3) were identical to the genes with significant methylation

158 differences between the origins at a respective temperature within laboratory samples (Table

159 2, see colour coding suppl. Table 5+7, sheets 'ST5 Origin' and 'ST7 Temperature').



160 Figure *3:* **VennEuler diagram showing the numbers of differentially methylated sites between**
161 **Helgoland and Spitsbergen when analysing field samples (blue), lab samples (orange), and all**
162 **(lab+field, grey) samples** (suppl. Tab. 1-3). The labels give the product of the genes that were
163 differentially methylated in the respective partitions. _(1) and _(2) depict a gene that showed
164 differential methylation at two different sites. For the exact site position in the respective gene, see
165 suppl. Table 5 ('ST5 Origins').

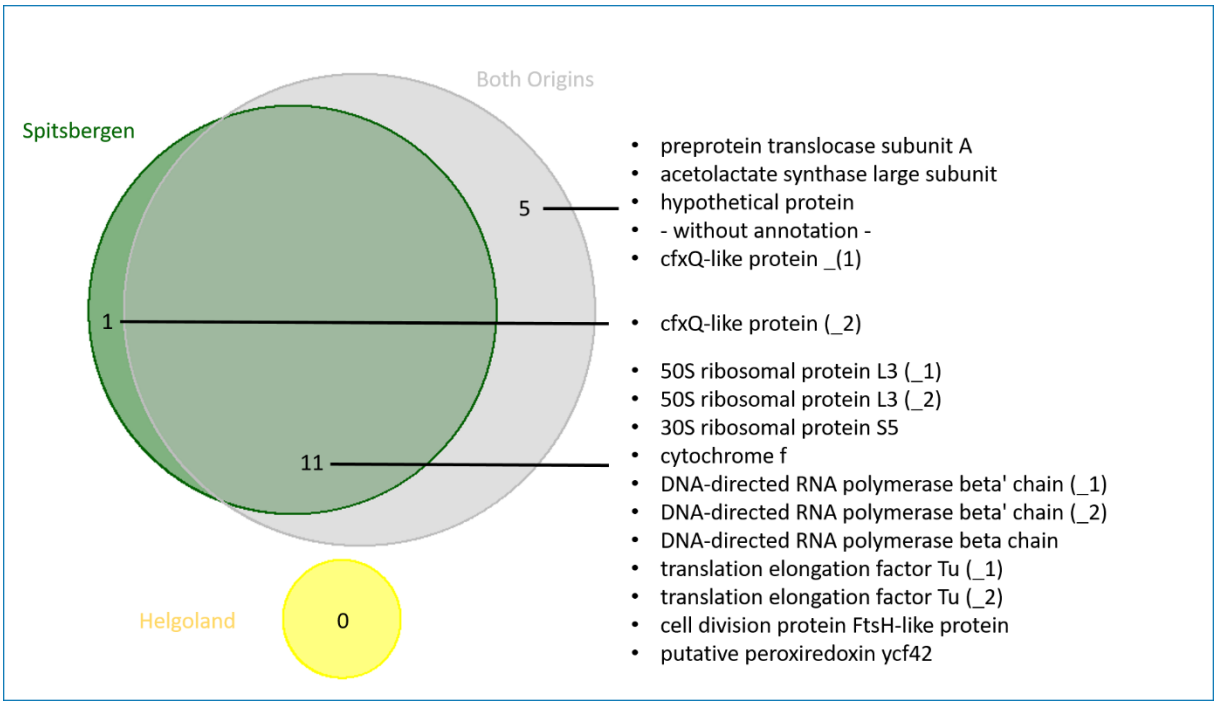
166

167 Of the 69 genes that were analysed comparing growth conditions (Fig. 4), 16 genes were

168 differentially methylated between laboratory and field samples, two of which were also

169 differentially methylated between origins (Fig. 3, Table 2). Many of those genes were

170 differentially methylated at two different positions (suppl. Table 6, sheet 'ST6 Cultivation').



171 Figure *4*: **VennEuler diagram showing the numbers of differentially methylated sites between lab**
172 **and field samples when analysing Helgoland samples (yellow), Spitsbergen samples (green), and the**
173 **samples from both origins together** (**grey**, suppl. Tab.4)**.** The labels give the product of the genes that
174 were differentially methylated in the respective partitions, with one gene without annotation record
175 at the ncbi database. _(1) and _(2) refer to a gene that showed differential methylation at two different
176 positions. For the exact position in the respective gene, see suppl. Table 6 ('ST6 Cultivation').

177

178 Two genes were differentially methylated between temperature treatments (Table 2), with

179 'acetolactate synthase large subunit' (QKE47546.1) at two comparisons: 5°C to 10 °C (padj <

180 0.0001), and 5 °C to 15°C (padj < 0.0017). All other significant differentially methylated genes

181 were found between origins at different temperatures, but only partially overlapped with

182 those obtained when analysing 'all samples' (Fig. 3, see colour codes in suppl. Tables 5 + 7

183 (sheets 'ST5 Origins' + 'ST7 Temperature')).

184

185 Table *2*: **All conditions that were found to be differentially methylated by the DESeq2 analysis when**
186 **analysing the different rearing temperatures of the laboratory samples.** Most significances were
187 detected between the origins at respective temperature (Helgoland vs Spitsbergen @ T5/10/15), but

188 only 3 of those were identical to those detected when all lab samples were being analysed as a group
189 (Fig. 3, Suppl. Table 7 ('ST7 Temperature')). However, genes QKE47546.1 and QKE47431.1 (bold letters)
190 showed significantly different methylation when analysing for temperature difference as factor (origin,
191 T vs T).

Comparison	**Gene Product**	**Gene ID**
5° vs 10° Helgoland	**acetolactate synthase large subunit**	**QKE47546.1**
5° vs 15° Helgoland	**acetolactate synthase large subunit**	**QKE47546.1**
5° vs 10° Spitsbergen	**photochlorophyllide reductase subunit B**	**QKE47431.1**
5° Helgoland vs Spitsbergen	photosystem I P700 apoprotein A2 _(1) ATP synthase CF1 alpha chain acetolactate synthase large subunit photochlorophyllide reductase subunit B	QKE47459.1 QKE47418.1 QKE47546.1 QKE47431.1
10° Helgoland vs Spitsbergen	photosystem I P700 apoprotein A2 _(1) ATP synthase CF1 alpha chain photosystem I assembly protein photochlorophyllide reductase subunit B	QKE47459.1 QKE47546.1 QKE47473.1 QKE47431.1
15° Helgoland vs Spitsbergen	photosystem I P700 apoprotein A2 _(1) ATP synthase CF1 alpha chain	QKE47459.1 QKE47546.1

192

193 None of the extracted GOterms were significantly enriched according to the 'topGO'
194 enrichment analysis.

195 *Analysis of Epigenetic Distance*

196 In both comparisons ('cultivation', 'origin'), the epigenetic distances between the samples
197 within respective groups were significantly different between groups (Fig. 5). For 'cultivation',
198 distances were significantly higher in laboratory samples than in field samples ($p < 0.0001$,
199 median field 153, lab 210), while for 'origin' the samples obtained from Spitsbergen showed
200 significantly higher epi-distances than Helgoland ($p < 0.0001$, median Helgoland 171,
201 Spitsbergen 190; suppl. Table 8).

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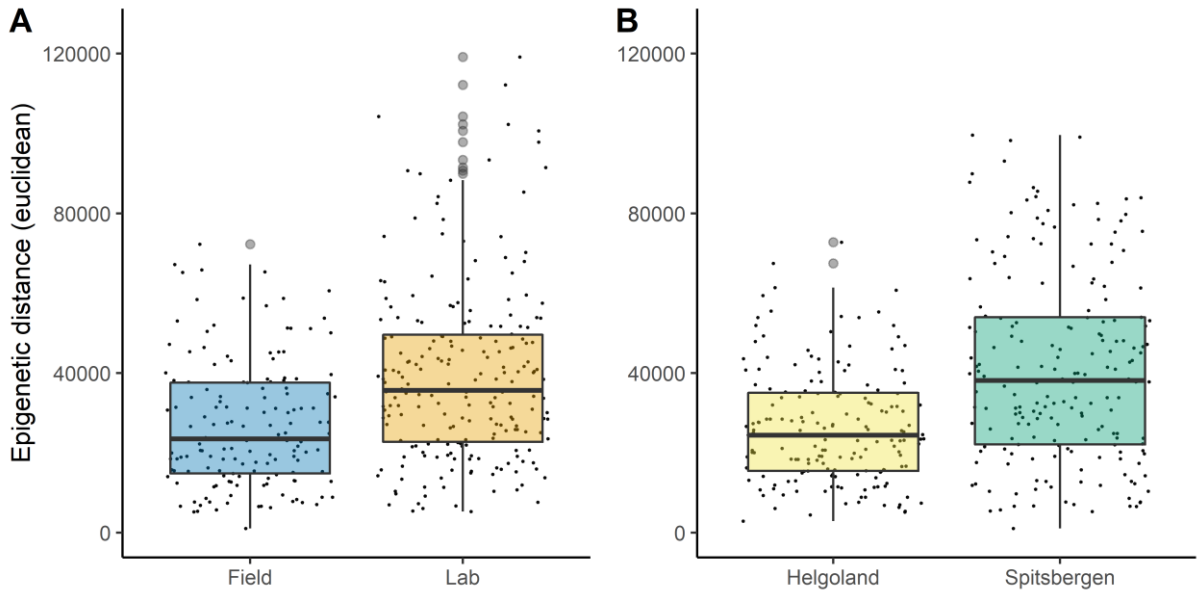

205 Fig. 5: **Epigenetic distance (Euclidean distance) for 'cultivation', Laboratory (orange) and Field (blue)**
206 **samples (A), and for 'origin', Helgoland (yellow) and Spitsbergen (green) samples (B).** The small black
207 dots depict the pairwise distances between the individual samples of each group (from the PCA, Fig.
208 2). The light grey dots are outliers detected by the boxplot script, but were included during epi-distance
209 analyses. The boxes represent the interquartile range (IQR) of the respective group, and the horizontal
210 line within the boxes gives the median Euclidean distance.

211

212 Discussion

213 Like recently reported for the kelp *Saccharina japonica* (Teng et al 2021), we detected
214 methylations in the chloroplast DNA of its congener *S. latissima* (Table 1). The chloroplast
215 methylome significantly differed between origins (Fig. 3, Table 2, Fig. 5 B), and growth
216 conditions (Fig. 4, Fig. 5 A), but temperature had a lesser impact than expected (Table 2).
217 Differentially methylated sites between origins (Spitsbergen or Helgoland) were located in
218 genes with functions related to photosynthesis. In contrast, differentially methylated sites
219 between growth conditions (laboratory or field) were almost exclusively located in genes
220 related to DNA replication and cell division (suppl. Table 5 + 6, sheets 'ST5 Origin' + 'ST6
221 Cultivation'; Fig. 3, Fig. 4). This suggests that the chloroplast methylome is origin-specific,

222 indicating an epigenetic memory, while showing capacity of rapid adaptation to shifts in
223 growth conditions via the epigenetic mechanism of cytosine methylation.

224 *Sample origin*

225 Population origin had a strong impact on the methylome according to PCA clusters (Fig. 2 B,
226 C, D), which was reflected in methylation levels, and methylated sites which significantly
227 differed between origins (Fig 1 A, C). Photosystem I (PSI) was the component most affected
228 by differences between the origins, as genes coding for varying aspects crucial to PSI
229 significantly differed at a total of four sites in three genes (Fig. 3, Table 2), with the strongest
230 effect on PSI P700 chlorophyll a apoprotein A2. In addition, the gene coding for apoprotein A2
231 in addition was one of two genes differentially methylated between the origins in all analysed
232 temperature conditions of the laboratory samples (Table 2, suppl. Table 7 sheet 'ST7
233 Temperature'). Therefore, the difference can be assumed to be solely accounted for by origin.
234 While methylation levels were significantly different only between the origins (Fig. 1), the
235 differences appear to be independent of the rearing condition (laboratory or wild), as most of
236 the differentially methylated genes between origins overlapped in field and lab samples (Fig.
237 3). Furthermore, the PCA indicated that origin plays a more pronounced role when analysing
238 laboratory and field samples separately (Fig. 2 C, D), while it is less pronounced when both
239 growth conditions were combined (Fig. 2 B). Hence, part of the methylome seems to be origin-
240 specific even after years of lab-cultivation. The differentially methylated genes were
241 exclusively related to functions of photosynthesis (Fig. 3), which likely can be explained by the
242 latitudinal differences in the light regimes. Light conditions in the High-Arctic are strongly
243 seasonal, with absence of photosynthetically active radiation (PAR) for at least three months
244 during the arctic winter (polar night), and continuous PAR during the arctic summer (polar
245 day). In boreal-temperate regions, PAR is available throughout the year. Whether or not the
246 differences observed here are of genetic nature cannot be assessed by our setup, but the

247 prevalence of the significant difference, detectable in the epigenetic signature that prevailed
248 through cultivation between origins, is a strong indicator for the eco-genotype approach
249 (Scheschonk et al 2022). This presumes that adaptation to a specific location is not solely a
250 matter of genetic nature (fixed only in the DNA), but that epigenetic mechanisms, especially
251 inheritable ones such as cytosine methylation, play an important role in local adaptation.
252 Considering methylation and gene expression were shown to be negatively correlated in the
253 *S. japonica* nuclear and chloroplast genes (Fan et al. 2020a; Teng et al. 2021) the (double-)
254 methylation of the gene coding for apoprotein A2 (Fig. 3, suppl. Table 5 sheet 'ST5 Origin'), for
255 example, which is one of the crucial components of PSI, can be presumed to have a direct
256 impact on the photosynthetic capacity which according to our results is likely to differ
257 between origins. Like with the nuclear genome (Scheschonk et al 2022) this has crucial
258 implications for cultivation processes as well as restoration efforts, as it again shows the
259 importance of suitable origin when considering local growth condition.

260 *Cultivation effects*

261 PCA showed that cultivation affected the methylome as strongly as origin (2 A, C). While lab
262 and field samples showed no significant differences in genome-wide methylation level and
263 number of methylated sites (Fig. 1), they displayed significantly different methylation levels
264 at 23.19 % of the sites (Fig. 4, suppl. Table 6 (sheet 'ST6 Cultivation'). Most (12 out of 16) of
265 the sites differentially methylated between rearing conditions had higher methylation levels
266 in the lab than in field samples (Suppl. Tab. 1). This suggests that those genes were higher
267 expressed in the field samples (Fan et al. 2020a; Teng et al. 2021). Unexpectedly, epigenetic
268 distance was higher in lab samples, even though they experience more uniform environmental
269 conditions than field samples. When translated to transcription, a higher epigenetic distance
270 is likely to result in more variable phenotypes. This might suggest that the kelp methylome
271 diversifies rather under stable lab conditions than under variable field conditions, at least

272 when said cultivation conditions were not previously encountered. Potential explanatory
273 factors cover the whole process of cultivation, from obtaining the sori, and securing the
274 zoospores, or handling during the fertilisation process, to culture conditions at any stage.

275 The lab cultures, which were grown from gametophytes that had been kept in culture for
276 about four years prior to the experiment (from 2014/2015 to 2018), experienced stable abiotic
277 factors such as nutrient availability, water current, light/dark cycle, and (before the
278 experimental treatments) constant temperature, and were protected from biotic stressors
279 such as grazing, pathogens, and competitors. Thus, cultivated / hatchery kelp might not have
280 activated those genes required to acclimate to environmental stressors encountered in the
281 wild due to (gametophyte) culture conditions, which might affect their performance when
282 deployed at sea for aquaculture or restoration purposes.

283 Nevertheless, origin had the strongest influence on the differences in cultivated samples at
284 gene level, as lab-field differences in methylation could only be detected in the Spitsbergen
285 population (Fig. 4, Table 2).

286 Another factor that might have impacted the methylomes of lab cultivated and wild kelp is
287 the age of the sporophytes. The field sporophytes were adult, while the lab samples were
288 juveniles with a size of approximately 12-15 cm. In plants, cytosine methylation levels tend to
289 increase with age, but these differences are most pronounced in meristematic areas
290 (Dubrovina and Kiselev 2016), which were avoided in the kelp samples. Because meristematic
291 regions were omitted, and there was no pattern consistent with older individuals (field
292 samples) having higher methylation levels in the chloroplast genome, the likelihood that the
293 results were affected by sporophyte age differences is low. Temperature as factor could be
294 shown to have an origin-specific impact on the methylome during cultivation (Table 2; see
295 next paragraph). However, which step, or factor, or combination thereof, during the

296 cultivation process is likely to have caused the increase in epigenetic distance will have to be
297 assessed in future studies. The difference in methylation between origins that persisted under
298 laboratory conditions might be due to epigenetic inheritance of a molecular memory of the
299 environment.

300 Whether or not genetic differences can result in the epigenetic differences that we observed
301 has yet to be determined. In plants and kelp, methylation can be present in CG, CHG, and CHH
302 sequence contexts, where H is any of the bases A, C, or T (Richards and Pigliucci 2020).
303 Cytosine methylation can be heritable across several generations (Lämke and Bäurle 2017),
304 hence is a likely candidate for rapid non-genetic but heritable local adaptation. The inheritance
305 of epigenetic traits induced by environmental factors has been repeatedly reported (Foust et
306 al. 2016; Gugger et al. 2016; Keller et al. 2016). This suggests that methylation differences can
307 remain stable across generations in cultivation, indicating that a transgenerational epigenetic
308 memory likely is present in the *S. latissima* chloroplast genome.

309 *Temperature and the methylome*

310 The temperature treatments did not form distinct clusters in the PCA. The cultivated *S.*
311 *latissimas*' chloroplast methylome, unlike the nuclear methylome, did not differ in
312 methylation levels and numbers of methylated sites in response to the rearing temperatures
313 of 5 °C, 10 °C and 15 °C. Significant differences solely caused by temperature were detected
314 in 2.9 % of the genes (Table 2). However, when analysing the origins at temperature level
315 (Table 2), most differences between origins found at discrete temperatures were in other
316 genes or at other gene sites than those detected for the origin analysis. This again suggests
317 the inheritance of an epigenetic memory in accordance with the eco-genotype approach
318 (Scheschonk et al. 2022), as the methylomes reacted origin-specific at discrete temperatures,
319 and it cannot be ruled out whether these epigenetic differences have a genetic base.

320 Increasing methylation levels have been shown to be positively correlated with heat stress
321 (Boyko et al., 2010; Correia et al., 2013). Heat stress is expected to excite a reaction in the
322 chloroplast due to the high temperature sensitivity of photosynthesis, but 15 °C, the highest
323 cultivation temperature, cannot be considered as 'heat', neither for chloroplast enzymes, nor
324 for *S. latissima* as organism. This temperature might have elicited a stress response in the
325 Spitsbergen samples, due to its presumed lack of epigenetic memory for this temperature. If
326 this were the case, however, the origins' response to 15°C during cultivation should have been
327 much stronger (more sites/ significant difference in methylation level) than to the lower
328 temperatures. However, 15°C was actually the temperature that had the least response in
329 terms of differential methylation (Table 2), hence this should not be considered the reason
330 here. A possible explanation for the overall low amount of (differentially) methylated sites
331 might be that the temperature exposure of the laboratory samples was not stressful for the
332 physiological processes regulated by the chloroplast genome. However, a reason for the
333 similarity of chloroplast DNA methylation at the different temperatures might be that
334 methylation sites affected by these temperatures are probably predominantly located in the
335 nucleus. In *Saccharina latissima*, it has been shown that temperature treatments affect the
336 nuclear methylome, and that differences in the methylome exist between populations from
337 different latitudes, suggesting that epigenetic modifications contribute to local adaptation
338 (Scheschonk et al. 2022). In addition, significant differences in the nuclear methylome were
339 found between wild and cultivated individuals (Scheschonk et al. 2022). Earlier research on *S.*
340 *latissima* suggests that short-term acclimation to different growth temperatures can lead to
341 changes in heat tolerance and affect photosynthetic performance (Davison et al. 1991).
342 Temperatures above approximately 20 °C generally result in poor growth, but at temperatures
343 below 15°C *S. latissima* shows a high capacity for acclimation (Andersen et al. 2013; Diehl and

344 Bischof 2021). Site-specific differences in circannual temperature may have contributed to the
345 different methylation patterns of populations from the high Arctic (Spitsbergen) and
346 temperate (Helgoland) regions. Here, heat stress may have occurred in the Helgoland
347 population during previous generations, as summer water temperatures can approach the
348 upper thermal tolerance of *S. latissima* (Bolton and Lüning 1982; Lüning 1984; Andersen et al.
349 2013). However, of the genes differentially methylated between origins, most were found at
350 discrete temperatures (5/10/15 °C), while not detectable when comparing the origins over all
351 lab samples.

352 *Methylation levels compared with the nuclear genome*

353 The methylation levels of sites in the chloroplast genome were ca. 100 times higher than levels
354 in the nuclear genome as documented by Scheschonk et al. (2022). In the nuclear genome,
355 the mean methylation level was 0.21% ± 0.13% SD (Scheschonk et al. 2022), while in the
356 chloroplast, the levels were 20.7% ± 2.2% for CH contexts and 24.8% ± 1.5% for CHG contexts
357 (Table 1). The higher methylation level in the chloroplast can be explained by the low numbers
358 of potential methylation sites in the chloroplast genome (Table 1). In comparison, in
359 *Saccharina japonica* the methylation level was reported as 1.4% in the nuclear genome (Fan
360 et al. 2020a) and in chloroplasts 17.1% at CG sites and 58.1% at CHG sites (Teng et al. 2021) of
361 sporophytes. Thus, the pattern of higher methylation in the chloroplast than the nuclear
362 genome is repeated in both *S. japonica* and *S. latissima*, with some dissimilarities that could
363 be caused by different sequencing methods and the inclusion of gametophytes in the studies
364 of *S. japonica* (Fan et al. 2020a; Teng et al. 2021). An example from land plants is in *Sequoia*
365 *sempervirens,* which has more similar methylation levels in its nuclear genome (7.0% - 8.6%)
366 and chloroplast genome (7.1% -7.6%), so in this plant the pattern of higher methylation in the
367 chloroplast is not present (Huang et al. 2012).

368 In the *S. latissima* nuclear genome, methylation was more site-specific in the population from
369 lower latitudes: the level of CG methylation was consistently higher, but the number of
370 methylated sites was lower in temperate Helgoland than in Arctic Spitsbergen (Scheschonk et
371 al. 2022). Such pattern of methylation becoming more site-specific has also been observed in
372 cyanobacteria in response to stress exposure (Hu et al. 2018). In contrast, in this study, the
373 chloroplast genome showed more methylated sites in Helgoland than in Spitsbergen and
374 higher methylation levels of CHG sites in Spitsbergen. Thus, the population specific pattern in
375 the nuclear methylome was not repeated in the chloroplast methylome.

376 In the chloroplast genome, there were no differences in methylated sites or methylation levels
377 between temperature treatments or between laboratory and field samples. In contrast, in the
378 nuclear genome, a response in methylation levels and numbers of methylated sites to warmer
379 temperature treatments was observed, especially in the Helgoland samples (Scheschonk et al.
380 2022). This difference suggests that the chloroplast genome responds differently to rising
381 temperatures than the nuclear genome, even though photosynthesis is highly sensitive to
382 temperature (Cen and Sage 2005).

383 However, the lack of statistically significant differences in the chloroplast genome as
384 compared with the nuclear genome may partly be explained by the smaller number of genes.
385 The chloroplast genome has a size of 130 kb and contains 139 protein-coding genes, three
386 rRNA genes and 29 tRNA genes (Fan et al. 2020b), while the nuclear genome has a size of 545
387 Mb and contains 18,733 protein-coding genes (Ye et al. 2015).

388 In the nuclear genome, most of the differentially methylated sites between lab and field
389 samples and between populations were in non-genic repetitive contexts (transposable
390 elements). In contrast, in the chloroplast genome, the differentially methylated sites were in
391 coding regions of genes, except for very few sites in a promoter region (Suppl. Tab. 1-4). The

392 higher number of methylated sites in coding regions in the chloroplast are likely related to
393 differences between chloroplast and nuclear genomes: chloroplast genomes are more
394 condensed and have far fewer noncoding regions than nuclear DNA (Clegg et al. 1994).

395 *Comparison with the congener species Saccharina japonica*

396 The number of methylated sites found in the chloroplast genome of *S. japonica* (4894 CG and
397 5807 CHG sites, Teng et al. 2021) was >20 times higher than in chloroplasts of *S. latissima*
398 (Suppl.Fig.7; 69 CG sites and 201 CHG sites, Table 1) identified in this study.

399 The low number of methylation sites is an expected outcome of the MethylRAD method. The
400 restriction enzyme used for digestion targets CCGG and CCHGG sites, which reduces the
401 number of potentially methylated sites compared to CG/CHG, and in addition targets the
402 specific bases within the degenerate adaptor endings 5'-NNNT-3' and 5'-NNNC-3', which only
403 constitute about one-eighth of all CG and CHG sites (Wang et al. 2015), while it does not target
404 CHH sites. CHH contexts were found to be the highest proportion in *S. japonica* based on
405 whole-genome bisulfite sequencing ('WGBS'; Fan et al. 2020a; Teng et al. 2021). Hence, the
406 methylation levels of the *S. latissima* chloroplast genome are expected to be much higher than
407 the mean number of covered sites we obtained, and are likely to be similar to that of *S.*
408 *japonica* when analysed with the same sequencing method (WGBS). However, as we found
409 both origin and cultivation to significantly influence differences in the methylome within the
410 same species, this should be taken into account for inter-species study comparisons as well.
411 Further, the life stage of the analysed samples plays a major role for methylation levels. In
412 both the nuclear, and chloroplast genome *Saccharina japonica* gametophytes were shown to
413 feature more methylation than sporophytes, and there is evidence suggesting that
414 methylation plays a role in regulating the kelp's life cycle (Fan et al. 2020a; Teng et al. 2021;
415 Yang et al. 2021).

416 ## Conclusion

417 Our results showed that sample origin and growth condition can strongly influence epigenetic
418 mechanisms, while temperature had a minor effect. As methylation differences in genes are
419 likely to trigger gene expression differences (Richards and Pigliucci 2020), this would directly
420 relate to eco-phenotype. The scale-up in cultivation of *Saccharina latissima* during the last
421 decade is likely to continue, and cultivation sites overall will likely shift more northward, in
422 accordance to the preferences of wild populations. The capacity of fast adaptation to changed
423 environmental factors, facilitated by epigenetic mechanisms, could prove particularly useful
424 in the face of rapid anthropogenic climate change. However, due to the high ecological and
425 economic value of this kelp, the implication of differences in methylation due to cultivation
426 relative to origin, and especially the possibility of chloroplast methylation influencing
427 photosynthetic capacity need to be further assessed. As most genes that were differentially
428 methylated in the chloroplast genome between origins contribute to aspects of
429 photosynthesis, an urgent question arising from our findings is whether differences in
430 chloroplast methylation impact photosynthetic capacity. Photosynthetic capacity is directly
431 linked to primary production, and the capability to build up and maintain biomass. This would
432 have implications for both cultivated and wild populations. In cultivars, the importance of
433 matching hatchery and cultivation conditions to origin has been implied by our results.
434 Furthermore, due to the significant differences observed in the methylomes of cultivated *S.*
435 *latissima*, it might be possible to detect an epigenetic 'fingerprint' of cultivated specimen in
436 the future, to detect escapees to the wild. Regarding the current trend in northward shift, the
437 further north populations of *Saccharina latissima* settle, the more pressure the months of
438 darkness during polar night will pose (Scheschonk et al. 2019). Photosynthetic pigments were
439 shown to be functional throughout the prolonged darkness of the Arctic winter (Scheschonk
440 et al 2019), indicating the chloroplasts of High Arctic specimen retained photosynthetic

441 capacity during polar night. In which context methylation of chloroplast DNA facilitated by
442 differences in the eco-phenotypes might have played a role in this context will be of interest,
443 especially regarding populations adapted to light conditions at more southern distributional
444 habitats, shifting northwards. In *Saccharina latissima*, phenotypic variation among
445 populations is often driven by phenotypic plasticity, and the species shows high plasticity in
446 response to changing abiotic factors (Bolton & Lüning 1982; Diehl and Bischof 2021). So far,
447 research on differences between origins in *S. latissima* has focused on genetic differentiation,
448 with significant genetic variation between populations (Møller Nielsen et al. 2016; Paulino et
449 al. 2016; Guzinski et al. 2020). Epigenetic differences have largely been unexplored as an
450 alternative source of molecular variation between *S. latissima* populations. The term 'eco-
451 phenotypes' has recently been introduced to include both epigenetic and genetic mechanisms
452 into the concept of heritable molecular variation that contributes to adaptation to local
453 environments and intraspecific variation between origins (Scheschonk et al. 2022). The
454 differences we observed between origins within the laboratory and the field samples indicate
455 that the eco-phenotype approach should be applied to the organellar chloroplast genome as
456 well. Furthermore, our data suggests that the influence of cultivation seems to be at least
457 partially origin-specific, and that the influence of cultivation and origin on the methylome are
458 interconnected. Epigenetic modifications have been found to be under some degree of
459 genetic control, but can also be triggered by environmental factors such as temperature
460 changes (Boyko et al. 2010; Correia et al. 2013). Hence, the role of genetic differences
461 controlling the epigenetic responses reported here needs to be assessed in further detail in
462 future studies.

463

464 # Materials and Methods

465 Laboratory cultures, field samples, and 'DNA- Extraction and MethlylRAD sequencing are
466 described in more detail in Scheschonk et al (2022). Changes and specific adjustments to the
467 present study were necessary during the bioinformatics and statistics part, and are detailed
468 below ('Bioinformatics and Statistics').

469 ## Laboratory cultures

470 As described in more detail in Scheschonk et al (2022), four cultures per origin (S1-S4, H1-H4;
471 technical replicates) were obtained from mono-parentally fertilised clonal gametophyte
472 cultures of *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders from
473 Helgoland, German Bight (Nordstrand, 54°11'18.9"N 7°54'14.1"E; HG1, HG2, HG3, HG4 in
474 culture since 2014) and Spitsbergen, Svalbard (Hansneset, Kongsfjorden, 78°59'26.0"N
475 11°58'42.3"E; SG1, SG2, SG3, SG4 in culture since 2015; see table Suppl. Table 9) were kept at
476 each of the three temperatures (5 °C, 10 °C, and 15 °C) under white light at 15 µmol m-2 s-1
477 (18 : 6 hours light : dark) from as early as initiation of fertilization. Only sporophytes with a
478 fresh weight exceeding 120 mg (without stipe) were taken for DNA extraction.

479 ## Field Samples

480 Adult sporophytes were sampled from Helgoland, German Bight (54°11'18.9"N 7°54'14.1"E),
481 and from Spitsbergen, Svalbard (78°59'26.0"N 11°58'42.3"E) at the same locations from which
482 the gametophytes for the laboratory grown sporophytes originated. From each sporophyte, 4
483 discs (ø=3 cm) were cut from the fronds, omitting the meristematic region, 'midsection', and
484 reproductive tissues. Samples from lab and field were dried in cellulose bags stored in silica.
485 In total 21 samples from the laboratory cultures, and 20 samples from the wild were analysed.
486 In contrast to the field samples, our laboratory samples were uniparentally fertilised.
487 However, given that chloroplast genes are transmitted solely from the maternal parent
488 (Kuroiwa 1991, in Nishimura 2010), the chloroplast genome inheritance is identical in

489 uniparentally and bi-parentally fertilised individuals. This circumstance enables a direct
490 comparison between the chloroplast methylomes of our cultured and wild samples.

491 Bioinformatics and Statistics of MethylRAD sequencing
492

493 Clean-up of raw data

494 DNA was extracted and MethylRAD sequencing libraries were prepared as described in detail
495 in Scheschonk et al (2022). The sequences were de-multiplexed by sample and quality
496 trimmed with TrimGalore! v 0.4.1
497 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Bases with Phred-
498 score <20 (--quality 20) were eliminated, the adapter sequence was removed (--stringency 3),
499 and the terminal 2bp (both ends) were removed to eliminate any artefacts at the ligation
500 position (--clip_R1 2 \ --three_prime_clip_R1 2). Reads were checked for fragment (over-)
501 representation, base AT CG content bias, and read length with FastQC v0.11.8 before and after
502 quality trimming.

503 In-silico digestion

504 For an overview of potential methylation sites, and as a backbone to map sequences to the
505 reference chloroplast genome for the *S. latissima* chloroplast (Fan et al. 2020b) was digested
506 *in silico* using the custom python script InSilicoTypeIIbDigestion_corrected.py
507 (http://marinetics.org/2017/04/11/REdigestions.html) and settings for simulating the
508 restriction enzyme FspEI. The *in silico* digestion found 270 recognition sites, where 69 were
509 CCGG recognition sites, 61 CCTGG sites, 77 CCTGT sites, and 63 CCAGG sites.

510 Mapping and annotation

511 The sequenced reads were mapped to the *in silico* digested reference genome of the *S.*
512 *latissima* chloroplast, combined with the *in silico* digested genome of the closely related
513 species *Saccharina japonica* to account for reads belonging to the nuclear genome and avoid
514 false mappings to the chloroplast genome for those reads. Reads were mapped using Burrows-

515 Wheeler Aligner (BWA Version: 0.7.17-r1188) (Li and Durbin 2010). Duplicate mappings were
516 excluded from further analyses by filtering out reads with a mapping quality <10 using
517 samtools (v 1.9). A count table was created listing the number of reads that mapped back to
518 each methylation site. For this, htseq-count (v 0.7.2) was called on each sample alignment
519 with *in silico* digested fragment in gff3 format as count features. An additional table was made
520 with counts normalized to 'reads per million (RPM) = (Reads mapped per site ×10^6)/(Total
521 number of mapped reads)' to account for varying sequencing depths.

522 Only methylated sites that were covered in at least one library with coverage >2 were kept in
523 count tables and RPM tables. The sites were annotated for their sequence context (CG or
524 CHG), genomic region, gene ID numbers, and gene functions based on the gff3 annotation file
525 for the *S. latissima* chloroplast genome from NCBI (Accession: MT151382.1) (Fan et al. 2020b).

526 Statistics

527 *Principal Component Analysis*

528 Differences in the chloroplast methylome were investigated with principal component
529 analysis (PCA) on RPM tables using the R package 'FactomineR' (Lê et al. 2008). Since lab and
530 field samples separated clearly into distinct groups in the PCA, their methylomes were
531 compared first for all samples, and then separated into origins (Helgoland and Spitsbergen).
532 Within the lab and field samples, and for all samples, we compared origins (Helgoland and
533 Spitsbergen). Within the lab samples, we compared between the treatment temperatures of
534 5°C, 10°C, and 15°C.

535 *Outlier detection*

536 The outlier function in the R package 'FactoInvestigate' (Thuleau & Husson, 2020) detected
537 two outliers, FH1 and FH9 (Helgoland field samples) in the PCA. The outliers showed few reads
538 mapping to the genome, few methylated sites, and high RPM values for some of the
539 methylated sites (Suppl. Fig. 2). Thus, FH1 and FH9 samples were excluded from all follow-up

540 analyses. No outliers were found in the Spitsbergen samples. As both outliers belonged to the
541 same group (Helgoland field samples), outlier removal led to a reduction in variance explained
542 by the first two principal components from 69.9% to 28.9% (Suppl. Figs. 2, 4, 5).

543 *Methylation levels*

544 We tested for differences in DNA methylation levels as RPM and for differences in the
545 numbers of methylated sites between populations, between treatment temperatures, and
546 between lab and field samples, using Wilcoxon rank sum tests. P-values were corrected with
547 the Benjamini-Hochberg method (Benjamini and Hochberg 1995) to control for the false
548 discovery rate, and results with adjusted p values padj <0.05 were considered significant. The
549 tests were performed using the R package 'rstatix' v0.7.0 (Kassambara, 2021).

550 We tested for correlation between the numbers of quality trimmed reads, and the numbers
551 of quality-trimmed reads that mapped back to the *Saccharina latissima* chloroplast genome
552 (Suppl. Fig. 4C, 5C, 4D, 5D).

553 *Differential methylation analysis*

554 We tested for significant differential methylation with the R package 'DESeq2' (Love et al.
555 2014) between 'Helgoland' *vs* 'Spitsbergen' in all samples ('All', or 'both locations'), and in only
556 field samples and only lab samples. Further, between 'field' *vs* 'lab' in all samples, and in only
557 Helgoland samples, and only Spitsbergen samples. We also compared pairwise between
558 temperature treatments: both origins combined (Helgoland + Spitsbergen 5 °C *vs* 10 °C; 5 °C
559 *vs* 15 °C; 10 °C *vs* 15 °C), separately for Helgoland and Spitsbergen (5 °C *vs* 10 °C; 5 °C *vs* 15 °C;
560 10 °C *vs* 15 °C), and for each temperature (Helgoland vs Spitsbergen @ 5 °C; 10 °C; 15 °C).
561 'DESeq2' normalised raw methylation counts before testing for differential methylation, and
562 corrected p-values with the Benjamini-Hochberg method for controlling false discovery rates
563 (Benjamini & Hochberg, 1995). Methylated sites with adjusted p-value padj < 0.05 were
564 considered significantly differentially methylated.

GO term analysis

Gene ontology (GO) terms for biological processes, cellular components, and molecular functions were extracted from the database https://www.uniprot.org/ using Gene ID numbers (Suppl. Tab. 1-4). The R package 'topGO' (Alexa & Rahnenführer, 2009) was used to test for enrichment of the GO terms.

Gene names and functions

Names and functions of differentially methylated genes were extracted from the chloroplast genome of *Saccharina latissima* published on NCBI (strain y-c14; https://www.ncbi.nlm.nih.gov/nuccore/MT151382.1?report=graph).

Analysis of epigenetic distance

Groups of the aspects 'cultivation' (lab vs field), and 'origin' (Helgoland vs Spitsbergen) were analysed for differences in epigenetic distances among the samples of each group. The Euclidean distances from the PCA including all samples were used for this. The Shapiro-Wilk indicated non-normal distribution for groups analysed for cultivation (p-value lab $p < 0.0001$, field $p < 0.0001$) and for origin (Helgoland $p < 0.0001$, Spitsbergen $p < 0.0001$), hence both tests for epigenetic distance were carried out applying the non-parametric Wilcox-rank-sum test (suppl. Table 8).

Data availability

Raw sequencer data of this study are available at NCBI SRA BioProject PRJNA809008.

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598 Author Contributions

599 LS designed and conducted the experiment, LS and MK extracted and sequenced the samples.
600 AN, LS and AJ analysed and interpreted the data. LS and AN wrote the manuscript. All authors
601 read and commented on previous versions of the manuscript, and approved the final version.

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763 Figure Legends

764 Figure *1*: **Significant differences in methylated sites and methylation level between conditions.**
765 Methylated CG sites (A, B), and methylation levels as reads per million (RPM) for CHG sites (C, D)
766 between populations and temperatures in laboratory samples (A, C) and compared between lab and
767 field samples (B, D). ns: not significant. Numbers at the bottom of the bars indicate sample size.
768 Significance codes after p-value correction (Benjamini-Hochberg) for multiple comparisons: '< 0.05': *,
769 '< 0.01': **, '< 0.001': ***, '< 0.0001': ****.

770

771 Figure *2*: **Principal Component Analysis for A: all samples, grouped by laboratory or field samples. B:**
772 **laboratory samples grouped by origin**. C: all samples, grouped by origin. D: field samples grouped by
773 origin. E: laboratory samples of both origins grouped by temperature treatment. Ellipses represent
774 95% confidence intervals around the group medians.

775

776 Figure *3:* **VennEuler diagram showing the numbers of differentially methylated sites between**
777 **Helgoland and Spitsbergen when analysing field samples (blue), lab samples (orange), and all**
778 **(lab+field, grey) samples** (suppl. Tab. 1-3). The labels give the product of the genes that were
779 differentially methylated in the respective partitions. _(1) and _(2) depict a gene that showed
780 differential methylation at two different sites. For the exact site position in the respective gene, see
781 suppl. Table 5 ('ST5 Origins').

782

783 Figure *4*: **VennEuler diagram showing the numbers of differentially methylated sites between lab**
784 **and field samples when analysing Helgoland samples (yellow), Spitsbergen samples (green), and the**
785 **samples from both origins together** (**grey**, suppl. Tab.4)**.** The labels give the product of the genes that
786 were differentially methylated in the respective partitions, with one gene without annotation record
787 at the ncbi database. _(1) and _(2) refer to a gene that showed differential methylation at two different
788 positions. For the exact position in the respective gene, see suppl. Table 6 ('ST6 Cultivation').

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790 Fig. 5: **Epigenetic distance (Euclidean distance) for 'cultivation', Laboratory (orange) and Field (blue)**
791 **samples (A), and for 'origin', Helgoland (yellow) and Spitsbergen (green) samples (B).** The small black
792 dots depict the pairwise distances between the individual samples of each group (from the PCA, Fig.
793 2). The light grey dots are outliers detected by the boxplot script, but were included during epi-distance
794 analyses. The boxes represent the interquartile range (IQR) of the respective group, and the horizontal
795 line within the boxes gives the median Euclidean distance.

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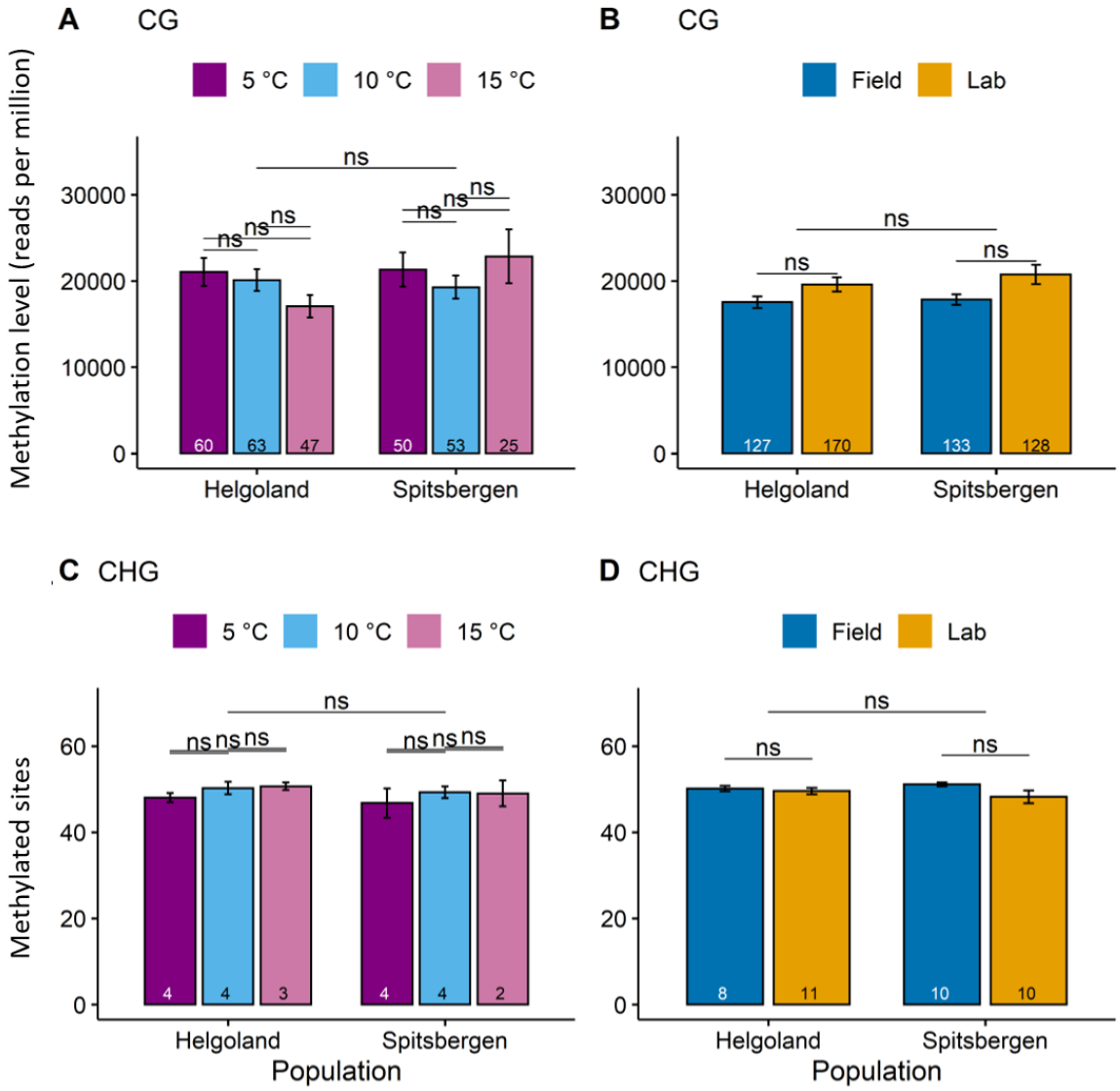
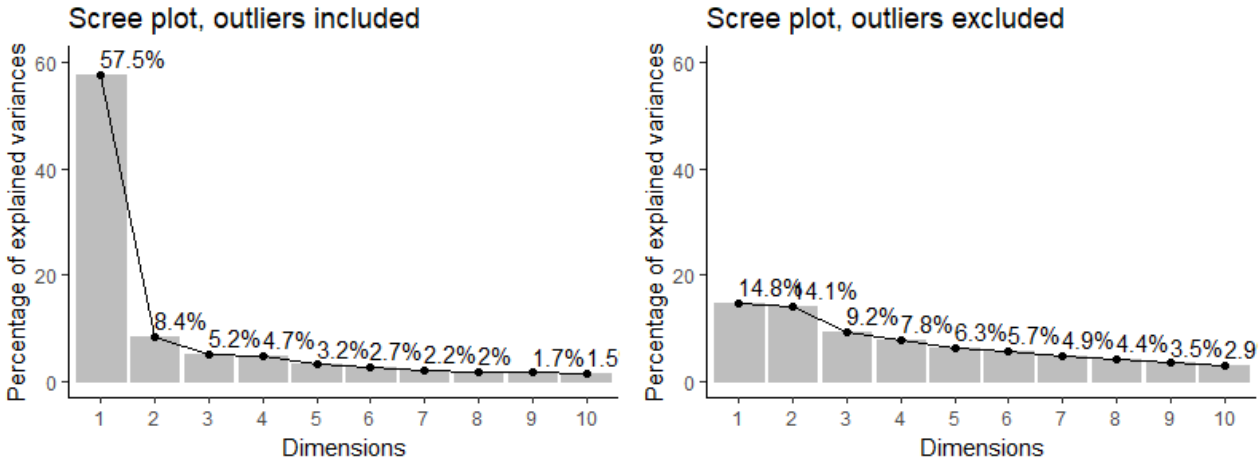
Supplementary

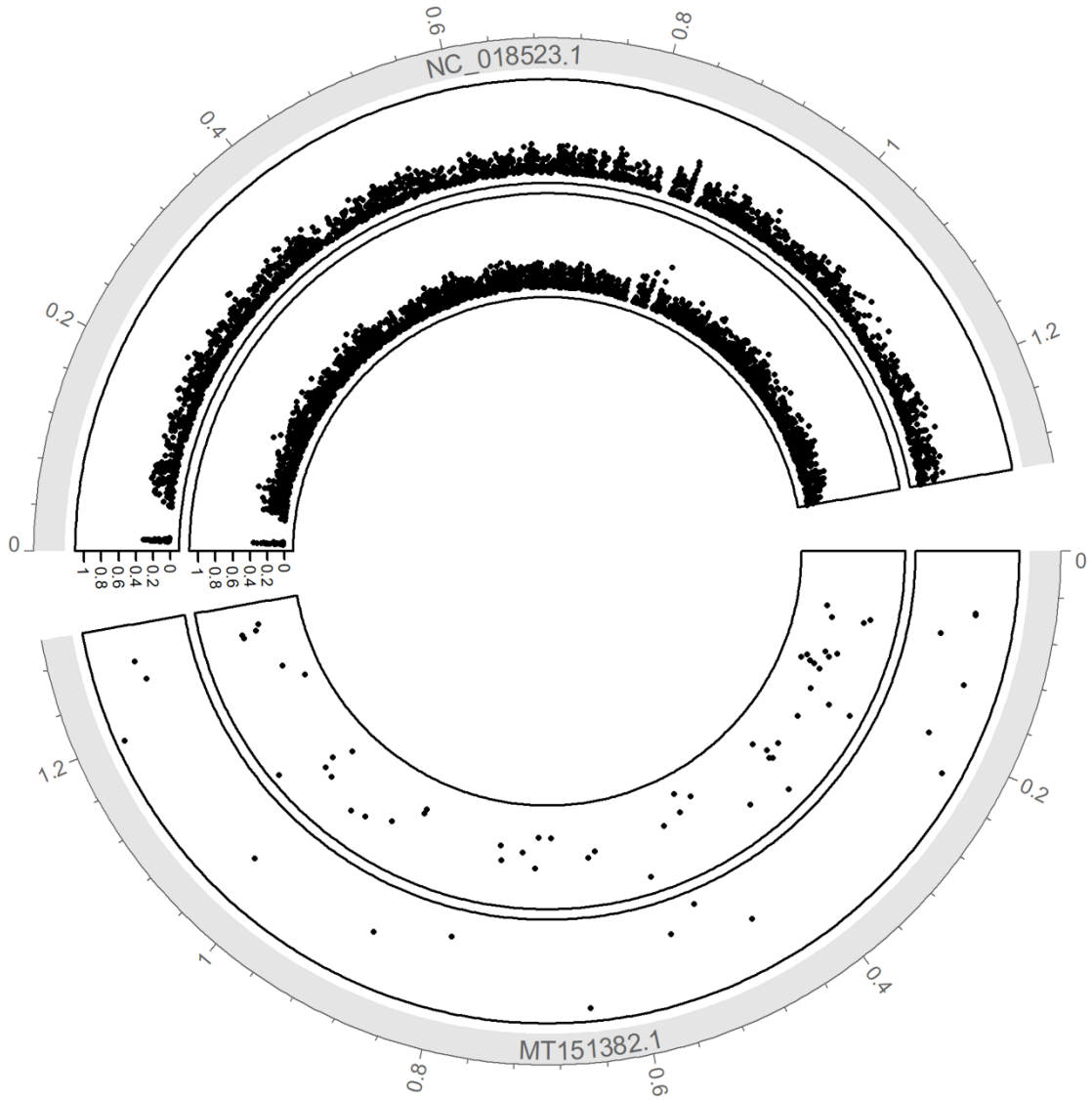
Suppl. Figures



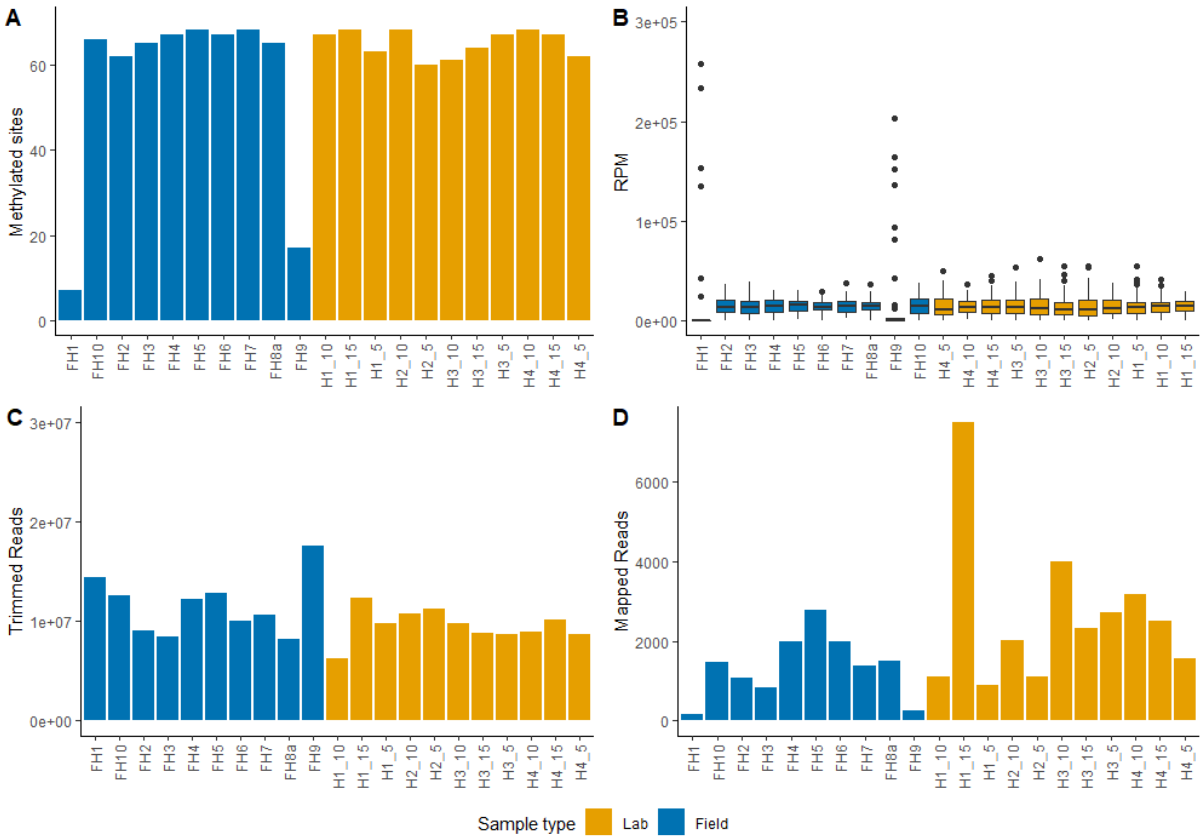
Suppl. Fig. *1*: D**ifferences in methylated sites and methylation level between conditions.** Methylation levels as reads per million (RPM) for CHG sites (A, B), and methylated CG sites (A, B) between populations and temperatures in laboratory samples (A, C) and compared between lab and field samples (B, D). ns: not significant. Numbers at the bottom of the bars indicate sample size.



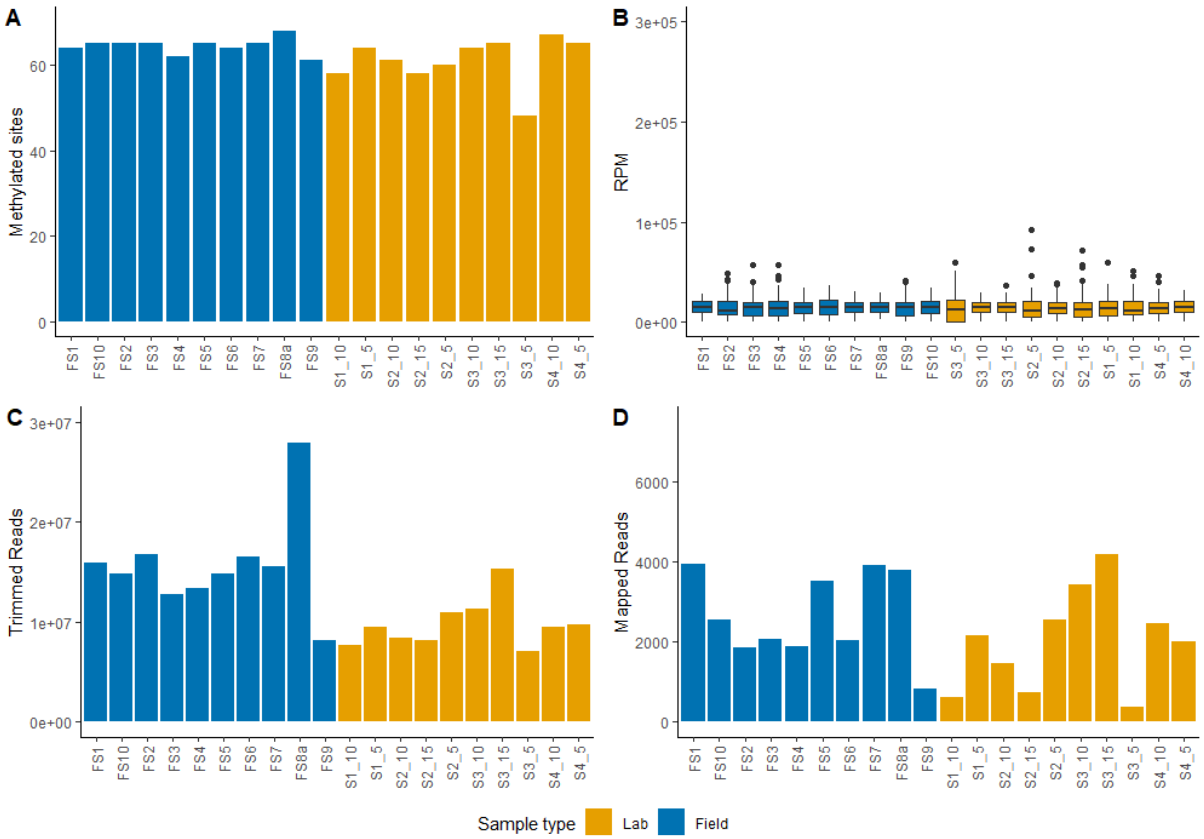
Suppl. Fig. *2*: **Scree plots showing the amount of variance explained by each dimension**. On the left including all samples, on the right when removing two outliers.



Suppl.Fig. *3:* **Overview of the chloroplast methylome in *S. latissima* (MT151382) and *S. Japonica* (NC_0198523.1).** *S. japonica* data from Teng et al. 2021. Numbers on the outside of the circle represent locations within the genomes. Numbers on y-axes show the methylation levels at each site. The inner circle shows CHG methylation, and the outer circle shows CG methylation. Numbers along the outer circles refer to megabases along the chloroplast genomes.



Suppl.Fig. *4*: **Sequencing output and methylation characteristics for each sample from Helgoland.** Plots were used for outlier detection for PCA. A: Number of methylated sites (with more than two reads) for each sample. B: Boxplot showing reads per million (RPM) for each sample, with each point representing one methylated site. C: The number of sequenced and quality-trimmed reads for each sample. D: Numbers of quality-trimmed reads that mapped back to the chloroplast genome of *Saccharina latissima*.



Suppl.Fig. *5*: **Sequencing output and methylation characteristics for each sample from Spitsbergen.** Plots were used for outlier detection for PCA. A: Number of methylated sites (with more than two reads) for each sample. B: Boxplot showing reads per million (RPM) for each sample, with each point representing one methylated site. C: The number of sequenced and quality-trimmed reads for each sample. D: Numbers of quality-trimmed reads that mapped back to the chloroplast genome of *Saccharina latissima*.

845 Suppl. Tables
846

Suppl. Tab. 1

Sites differentially methylated (p < 0.05) between Helgoland and Spitsbergen populations when including all samples in the comparison. Positive Log2FC values indicate increased methylation in Spitsbergen compared to Helgoland, while negative values indicate higher methylation in Helgoland samples.

Location	Log2FC	Log2FC std error	Adjusted p value	Gene ID	Gene name	Region	Product	Sequence context
21922-21953	1.04	0.196	2.66E-06	QKE47428.1	rpoB	gene	DNA-directed RNA polymerase beta chain	CHG
68544-68575	0.933	0.302	0.0274	QKE47473.1	ycf3	gene	photosystem I assembly protein	CHG
39358-39389	0.456	0.159	0.0475	QKE47440.1	groEL	gene	60 kDa chaperonin	CHG
51728-51760	-4.00	0.903	0.000164	QKE47458.1	psaA	promoter	photosystem I P700 apoprotein A1	CG
9045-9077	-4.17	0.77	2.05E-06	QKE47418.1	atpA	gene	ATP synthase CF1 alpha chain	CG
55344-55376	-4.96	0.644	9.14E-13	QKE47459.1	psaB	gene	photosystem I P700 apoprotein A2	CG

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Suppl. Tab. 2

Sites with differential DNA methylation (p < 0.05) between Helgoland and Spitsbergen populations when comparing only field samples. Positive Log2FC values indicate increased methylation in Spitsbergen compared to Helgoland, while negative values indicate higher methylation in Helgoland samples.

Location	Log2FC	Log2FC std error	Adjusted p value	Gene ID	Gene name	Region	Product	Sequence context
21922-21953	0.867	0.256	0.0117	QKE47428.1	rpoB	gene	DNA-directed RNA polymerase beta chain	CHG
39358-39389	0.776	0.222	0.0104	QKE47440.1	groEL	gene	60 kDa chaperonin	CHG
75619-75650	0.727	0.206	0.0104	QKE47479.1	cbbX	gene	cfxQ-like protein	CHG
55567-55598	-0.528	0.173	0.0259	QKE47459.1	psaB	gene	photosystem I P700 apoprotein A2	CHG
51728-51760	-3.15	0.994	0.0209	QKE47458.1	psaA	promoter	photosystem I P700 apoprotein A1	CG
55344-55376	-4.49	1.21	0.0104	QKE47459.1	psaB	gene	photosystem I P700 apoprotein A2	CG

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Suppl. Tab. 3

Sites with differential DNA methylation ($p < 0.05$) between samples originating from Helgoland or Spitsbergen when comparing only laboratory samples. Positive Log2FC values indicate increased methylation in Spitsbergen compared to Helgoland, while negative values indicate higher methylation in Helgoland samples.

Location	Log2FC	Log2FC std error	Adjusted p value	Gene ID	Gene name	Region	Product	Sequence context
68544-68575	1.43	0.457	0.023	QKE47473.1	ycf3	gene	photosystem I assembly protein	CHG
21922-21953	1.18	0.285	0.000824	QKE47428.1	rpoB	gene	DNA-directed RNA polymerase beta chain	CHG
9045-9077	-5.24	0.701	2.71E-12	QKE47418.1	atpA	gene	ATP synthase CF1 alpha chain	CG
51728-51760	-5.24	1.43	0.00438	QKE47458.1	psaA	promoter	photosystem I P700 apoprotein A1	CG
55344-55376	-5.68	0.639	4.27E-17	QKE47459.1	psaB	gene	photosystem I P700 apoprotein A2	CG

Suppl. Tab. 4

Sites differentially methylated (p < 0.05) between laboratory and field samples. Positive Log2FC values indicate higher methylation in laboratory samples compared to field samples.

Location	Log2FC	Log2FC std error	Adjusted p value	Gene ID	Gene name	Region	Product	Sequence context
88361-88392	2.27	0.479	0.000137	QKE47493.1	rpl3	gene	50S ribosomal protein L3	CHG
15235-15266	1.16	0.282	0.000659	QKE47426.1	rpoC2	gene	DNA-directed RNA polymerase beta' chain	CHG
88373-88404	0.987	0.298	0.00741	QKE47493.1	rpl3	gene	50S ribosomal protein L3	CHG
17309-17340	0.952	0.31	0.0124	QKE47426.1	rpoC2	gene	DNA-directed RNA polymerase beta' chain	CHG
64927-64959	0.752	0.173	0.000294	QKE47469.1	petA	gene	cytochrome f	CG
34231-34262	0.598	0.198	0.0134	QKE47436.1	secA	promoter	preprotein translocase subunit A	CHG
102711-102742	0.591	0.172	0.00654	QKE47520.1	tufA	gene	translation elongation factor Tu	CHG
124685-124717	0.561	0.181	0.0123	QKE47544.1	ycf39	gene	NmrA domain-containing protein	CG
46721-46753	0.551	0.195	0.0237	QKE47451.1	ycf42	gene	putative peroxiredoxin ycf42	CG
32980-33011	0.514	0.143	0.00416	QKE47436.1	secA	gene	preprotein translocase subunit A	CHG
102611-102643	0.49	0.175	0.0241	QKE47520.1	tufA	gene	translation elongation factor Tu	CG
22542-22574	0.439	0.161	0.0264	QKE47428.1	rpoB	gene	DNA-directed RNA polymerase beta chain	CG
75988-76019	-0.435	0.158	0.0253	QKE47479.1	cbbX	gene	cfxQ-like protein	CHG
104220-104251	-0.567	0.173	0.00741	QKE47523.1	ftsH	gene	ATP-dependent zinc metalloprotease FtsH	CHG
127333-127364	-0.641	0.191	0.00741	QKE47546.1	ilvB	gene	acetolactate synthase large subunit	CHG
95574-95605	-0.728	0.16	0.000183	QKE47509.1	rps5	gene	30S ribosomal protein S5	CHG

Suppl.Table *5* see excel file 'SupplTab5_6_7_Fig3_4_Tab2' sheet 'ST5 Origins'
Suppl.Table *6* see excel file 'SupplTab5_6_7_Fig3_4_Tab2' sheet 'ST6 Cultivation'
Suppl.Table *7* see excel file 'SupplTab5_6_7_Fig3_4_Tab2' sheet 'ST7 Temperature'

Suppl.Table *8:* Statistics results of the epigenetic distance analysis.

Epidistance field/lab test results				**Euclidean distance**	
Wilcoxon rank sum test		*statistic*	10499		**Mean dist.**
		p	0.0000000173	*Field*	153
		p.signif	****	*Lab*	219
Shapiro-Wilks test	*Field*	*statistic (W)*	0.945		
		p	0.00000106		
	Lab	*statistic (W)*	0.932		
		p	0.0000000264		
Epidistance Helgoland/Spitsbergen test results				**Euclidean distance**	
Wilcoxon rank sum test		*statistic*	10330		**Mean dist.**
		p	0.00000000231	*Helgoland*	171
		p.signif	****	*Spitsbergen*	190
Shapiro-Wilks test	*Helgoland*	*statistic (W)*	0.965		
		p	0.0000305		
	Spitsbergen	*statistic (W)*	0.957		
		p	0.0000172		

Suppl.Table 9: Fertilisation scheme of the laboratory male (♂) and female (♀) gametophyte cultures from Helgoland (HG1-4) and Spitsbergen (SG1-4), resulting in the cultures (Helgoland (H1-4) and Spitsbergen (S1-4) raised under laboratory conditions.

Gametophyte Cultures	HG2 ♀ X HG2 ♂	HG3 ♀ X HG3 ♂	HG4 ♀ X HG4 ♂	SG1 ♀ X SG1 ♂	SG2 ♀ X SG2 ♂	SG3 ♀ X SG3 ♂	SG34 ♀ X SG4 ♂
Sporophyte Culture	H2	H3	H4	S1	S2	S3	S4