Application of QTL and GWAS approaches in Saccharina latissima

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GENetic diversity exploitation for Innovative macro-ALGal biorefinery

Deliverable D2.3

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Planned delivery date (as in DoA): June 2021

Actual submission date: August 2021
Workpackage: WP2

Workpackage leader: CNRS

Deliverable leader: CNRS
Version: 2.0 (26/11/2021)

Project co-funded by the European Commission within the Horizon 2020 Programme (2014 - 2020)	
Dissemination Level	
PU Public	**X**
CI Classified, as referred to Commission Decision 2001/844/EC	
CO Confidential, only for members of the consortium (including the Commission Services)	

Research and Innovation action: GA no. 727892

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Project and work package:

Genialg, work package 2

Participants:

CNRS (lead), SAMS, NUIG, all other partners

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Executive summary

This deliverable describes work carried out as part of work package 2 of the Genialg project aimed at demonstrating proof of concept for the application of genome-based breeding approaches to the cultivated kelp *Saccharina latissima*. The two main approaches applied were identification of quantitative trait loci (QTLs) and genome-wide association studies (GWAS). QTL approaches focused on an F2 family generated by crossing two parental strains for north and south Brittany. Segregating families were generated for both the sporophyte and the gametophyte generations and, for both populations, both genotype and phenotype data was collected. This allowed the establishment of genetic maps based on the two different life cycle generations and searches for QTLs during both generations. The GWAS approach was carried out over two successive seasons, using a total of 494 *S. latissima* individuals.

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Introduction

This deliverable is associated with work package 2, task 3.1 "Application of QTL and GWAS approaches for strain improvement". The aim of this task was to provide proof of concept of the feasibility of applying modern, marker-based breeding strategies to macroalgal breeding. The selected target species was *Saccharina latissima* and the breeding approaches applied were quantitative trait locus (QTL) analysis and a genome-wide association study (GWAS). The

QTL approach was applied to two sets of progeny representing the two generations of the life cycle, sporophytes and gametophytes (both derived from the same F1 sporophyte parent). The objective, in working with these two families, was to determine whether both generations could be used for QTL identification and to investigate whether characteristics detected during the gametophyte generation might be relevant to breeding aimed at the sporophyte generation. The GWAS approach, which used strains collected from sites that represent the broad expanse of European *S. latissima* populations, aimed to analyse similar phenotypes to those analysed for the sporophyte QTL family in order to permit a comparison of the two approaches.

Establishment of a segregating sporophyte F2 family

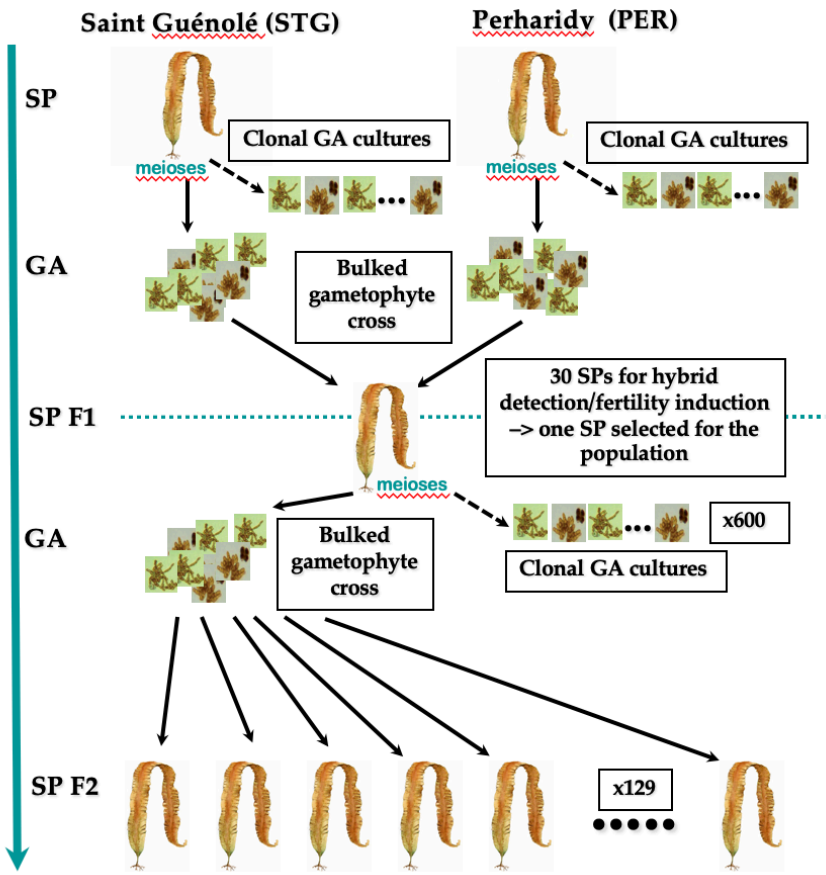

Figure 1. Breeding strategy for the generation of a segregating family of *S. latissima* sporophytes for QTL mapping.

Both the sporophyte (Figure 1) and gametophyte (see below, Figure 8) QTL families were derived from a cross between two strains of *S. latissima* collected at Perharidy (near Roscoff,

north Finistère, Brittany, France; January 2016; strain SLPER139) and Saint Guenolé (south Finistère, Brittany, France; October 2015; strain SLSTG055)[1]. Mixes of male and female gametophytes, derived by sporulation of each of the two strains, were combined in a bulk gametophyte cross (February 2016). The F1 sporophyte progeny from this cross were cultivated on an open sea rope system near Roscoff in the Morlaix bay (from April 2016). The F1 sporophytes were genotyped using microsatellite markers in order to select a hybrid F1 individual (strain F1.27.2).

The hybrid F1 individual (strain F1.27.2) was sporulated to produce a mix of male and female gametophytes. To create the F2 sporophyte family, a bulk gametophyte cross was carried out and the sporophytes were allowed to attach to three different attachment strings by incubating with the string in an aquarium system. The F2 sporophyte progeny from this cross were then cultivated on an open sea rope system at the same site (2017) by attaching the three strings to a single rope. The positions of the individuals on the strings and on the rope were recorded. Morphometric measurements were made during growth (sporophyte total length, including stipe, and width) and the presence of epiphytes noted (June and July 2017).

The F2 sporophyte family (129 individuals) were harvested and stored overnight (about 12 hours) in net bags in the dark in a outside container immersed in flowing natural seawater (at about 13°C) at the Roscoff aquarium (October 2017). Note that this period of storage is likely to have affected metabolic parameters (degradation of carbon reserves and possibly also proteolysis, reduced nitrate assimilation due to reduced chloroplastic glutamine synthetase activity and feedback on nitrate reductase activity).

Phenotyping of the F2 sporophyte family

These 129 F2 individuals were individually photographed and the following morphometric measurements recorded (Figure 2):

- Total length (without holdfast, which had been removed)
- Stipe length
- Total blade length

[1] Note that crosses were also carried out between more distantly related strains at the same time but the progeny of these crosses were lost during open sea culture.

- Blade width (at widest point)
- Blade length without epiphytes
- Length of the part of the blade without epiphytes
- Length of the part of the blade with few epiphytes
- Length of the part of the blade with many epiphytes

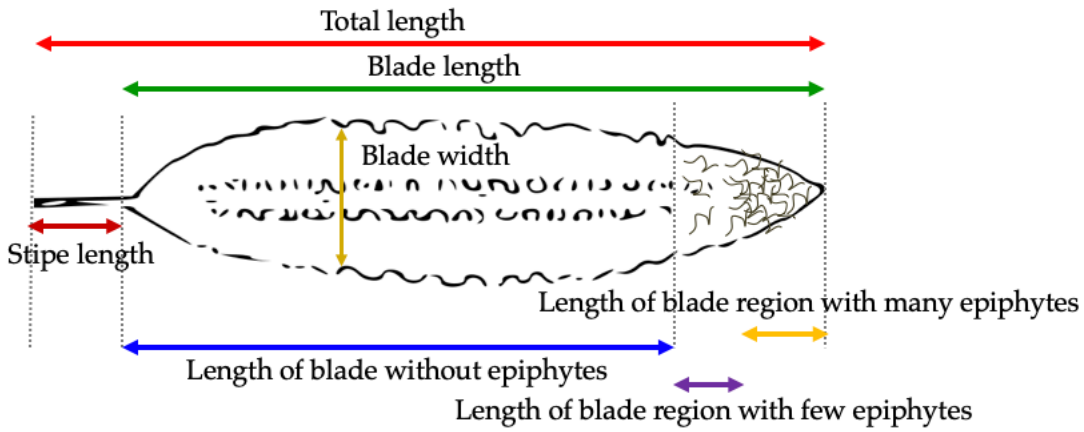

Figure 2. Morphometric measurements carried out on the *S. latissima* family.

Large (34 mm diameter) and small (17 mm diameter) tissue disks were cut from the blades (Figure 3) as follows (when blades were small, fewer disks were taken):

- 18 small disks for a stress experiment.
- 3 small disks for fucan, alginate, laminarin, mannitol and phenolic measurements
- 3 large disks for induction of sporogenesis
- 3 small disks from meristem region for growth experiments
- 10 small disks for later biochemical analyses and back-up (RNA, DNA)
- The remainder of blade was harvested for cell wall analyses

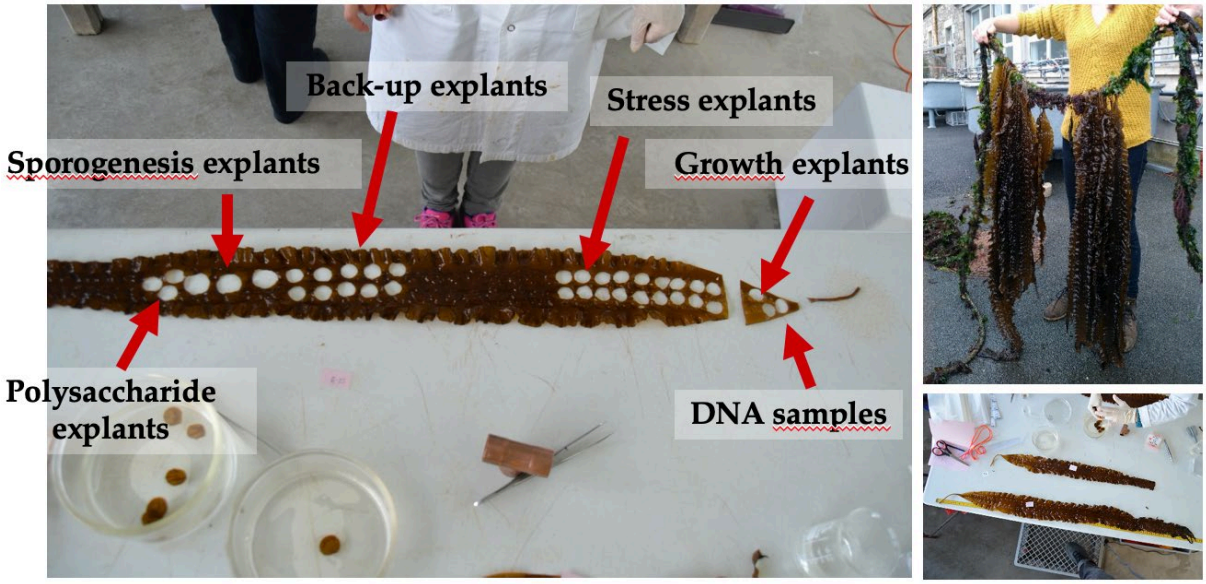

Figure 3. Harvesting and sampling of the *S. latissima* family. (left) Collection of tissue disks. (above right) Harvested thalli. (lower right) Photographing of harvested thalli.

For disk fresh weight (FW) / dry weight (DW) measurements, 1 large disk was frozen at -80°C for each F2 individual and then weighed after removal from the freezer and a second time after lyophilisation. In addition, DW/FW was calculated as a percent.

Growth experiment

For each F2 individual, three small disks were placed in PES in 55 mm Petri dishes and incubated at 11°C under white fluorescent light of 10-30 mol m^2s^1 photon fluence rate, 12 hours light: 12 hours dark (culture cabinet) for 45 days. The disks approximately doubled in size but it was not possible to measure area increase because they grew in a bowl shape. The wet weight was measured at the end of the growth period but this did not allow growth to be measured because the initial weight had not been recorded. To nonetheless recover a measurement for the QTL analysis, the weight of the heaviest disk of the three was recorded (max_weight) based on the logic that the heaviest disk should be the one that corresponded with the position of the fastest growing region of the meristem.

Resistance to temperature stress

The protocol planned for the temperature stress experiment involved a stress treatment at 21°C for 2 days followed by a recovery period at the control temperature of 13°C (Figure 4).

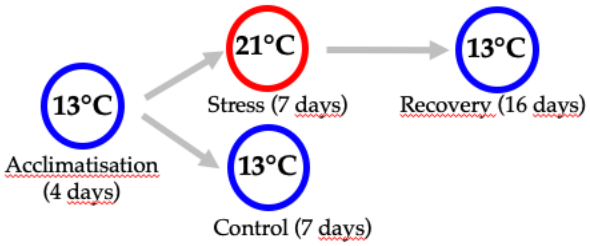

Figure 4. Planned protocol used for the temperature stress experiment.

However, during the experiment, Fv/Fm measurements indicated that the disks were not stressed after 2 days at 21°C so a stress of 2 days at 23°C was applied to a parallel set of disks that had been maintained at 13°C (Figure 5). Fv/Fm measurements subsequently showed that the disks at 21°C were stressed after 7 days at this temperature and, as it had not been possible to include a recovery phase for the 23°C stress treatment, the latter stress was not analysed in detail. The analysis therefore focused on the 7 days stress at 21°C.

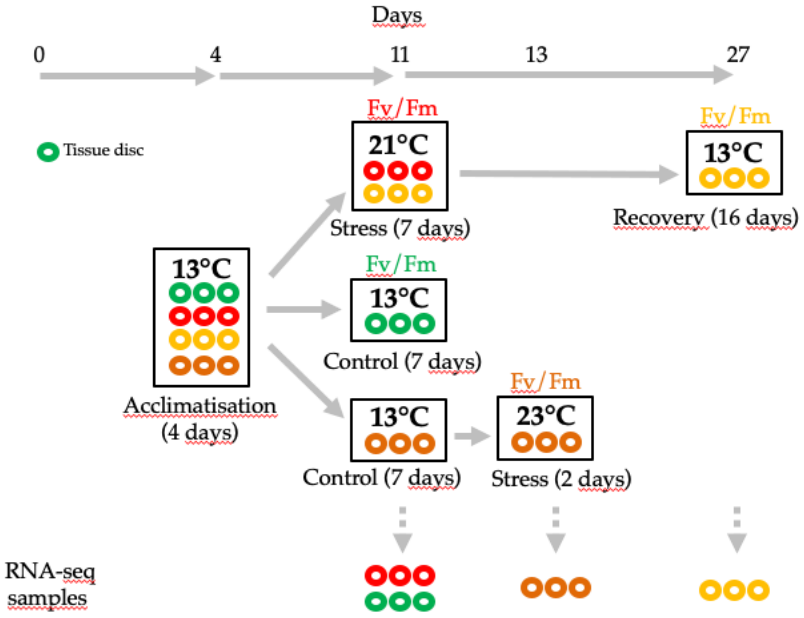

Figure 5. Actual protocol used for the temperature stress experiment.

The experiment was carried out as follows: 12 small tissue disks per F2 individual were acclimatised for 4 days at 13°C and then either incubated at 13°C for 7 days (6 disks, control)

or heat stressed at 21°C for 7 days (6 disks, stress). The heat stressed disks were either harvest directly after heat stress (3 triplicate disks, 21°C stress) or incubated at 13°C for 16 days to recover before harvesting (3 triplicate disks, 21°C stress plus recovery). The "control" disks were either harvested after the 7 day, 13°C treatment (3 triplicate disks, control) or transferred to 23°C for 2 days (3 triplicate disks, 23°C stress). Fv/Fm measurements were made after heat stress and after the recovery period (the colours indicate which disks were analysed at which timepoint).

RNA-seq data will be generated for outlier F2 individuals based on the stress experiment. The sequencing will be carried out by Genoscope as part of the Phaeoexplorer project. For each F2 outlier individual, RNA will be extracted from 3 pooled disks after 4 days acclimatisation at 13°C and from 3 pooled disks after 4 days acclimatisation at 13°C followed by 7 days of heat stress at 21°C. The selected outlier individuals are:

- 95, 96, 122 (Resilient, i.e. sensitive to stress but recover)
- 1, 31, 35 (Sensitive ++, i.e. Fv/Fm<600 after stress, dead or moribund after recovery period)
- 10, 42, 44 (Tolerant, i.e. Fv/Fm stable throughout the experiment)

A temperature stress experiment was also carried out using a conductivity test to detect release of cellular electrolytes as an alternative to the above approach based on Fv/Fm measurements. Small tissue disks (3 per treatment, 6 in all per F2 individual) were incubated at 13°C for 24h in PES (10-30 mol m^2s^1 photon fluence rate white fluorescent light 12h:12h light:dark cycle), then 24h in 50% PES : 50% 1M sorbitol, then 24h in 1M sorbitol, before being incubated at either 13°C (control) or at 21°C (stress) for 24 hours. Note that the osmolarity of 1M sorbitol is similar to seawater but conductivity (about 40µS/cm) is negligeable. Electrolyte leakage was estimated by measuring the conductivity after each treatment (Cond1), then freezing the disks at -80°C (which kills all the cells causing maximum electrolyte leakage) and then re-measuring electrolyte leakage (CondTOT). Percent electrolyte loss before freezing (Cond% = (Cond1/CondTOT)*100) provides an estimate of the extent to which the cells of the disk are stressed under each condition. An average value of Cond% was calculated for each condition and the difference between the averages for disks incubated at 21°C vs 13°c (meanCond%stress-meanCond%control) provided a measurement of the effect of the stress (compared to the control) on electrolyte leakage (a high value indicates high sensitivity). The values for the conductivity tests were not correlated with those obtained for Fv/Fm possibly due

to stress effects of the sorbitol incubation so these data should be treated with care and will probably not be included in the final study.

Metabolic parameters

One blade disk for each of the F2 individuals was lyophilised and ground to a powder in Roscoff and then sent to NUIG in Galway. Duplicate assays (technical duplicates) for nitrates, ammonium, proteins and amino acids were carried out with about 5 mg of powder from each sample (3-8 mg). Soluble metabolites were extracted with sequential hot ethanolic extraction (100%, 80% and 50% EtOH with pooling of the three different extracts in the same tube) and the insoluble pellet was resuspended in NaOH. The redissolved insoluble pellet was used for the determination of protein content. The other assays used the pooled hot ethanolic extracts. No nitrite was detected in the samples. No soluble sugars (glucose, fructose, sucrose et rhamnose) were detected in the samples (probably due to dark storage).

Analysis of cell wall composition

These analyses used the blade material that remained after the tissue disks had been removed from the F2 individual blades. Alcohol-insoluble residues (AIRs) containing total cell walls were prepared (Figure 6). Briefly, the individuals were air dried in a ventilated oven at 50°C and ground to a fine powder using a Retsch MM200 shaking metal bead system. Quadruplicate samples of 20 mg of ground powder were washed in 70% ethanol at 40°C, washed in 96% ethanol, then washed in 1:1 chloroform/methanol and washed with acetone. Each extraction was carried out three times for 3 min each time (12 extractions in total). These AIR samples were then dried in a ventilated oven.

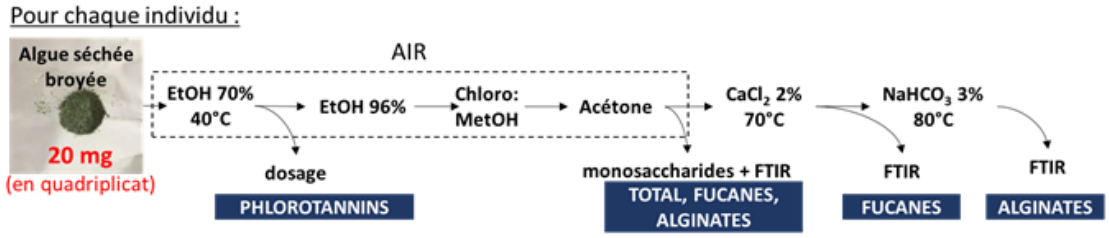

Figure 6. Protocol for the preparation of extracts for FTIR analysis and phlorotannin assays.

Regarding the monosaccharide content of those walls, a preliminary series of analyses was carried out at Lille University (PAGés facility) using AIR material from nine "outlier" individuals that had showed extreme morphometric, stress or protein content phenotypes:

- F2 individual number 38 - very stress sensitive
- F2 individual number 42 - very stress resistant
- F2 individual number 44 - very stress resistant
- F2 individual number 58 - very low protein content
- F2 individual number 59 - very low protein content
- F2 individual number 64 - very high protein content
- F2 individual number 68 - very large thallus
- F2 individual number 71 - very stress sensitive
- F2 individual number 85 - very large thallus

Sugars were analysed from AIRs using a methanolysis treatment for polysaccharide hydrolysis prior to the sugar assay. These assays used the trimethylsilyl derivative method (gas chromatography mass spectrometry) including standard ranges for nine monosaccharides (Figure 7). Analyses of monosaccharide (fucose, xylose, mannuronic acid, guluronic acid, galactose and glucose) content indicated very low variance across the nine individuals (the alginate components mannuronic acid and guluronic acid were the most abundant monosaccharides). In contrast, a large variation in the quantity of sugars (from 154 to 341 µg/mg) was observed but this may have been due variation in the efficiency of hydrolysis priori to measurement. Note that monosaccharide content is best analysed as a percentage of total sugars (e.g. % mg fucose/mg total monosaccharides).

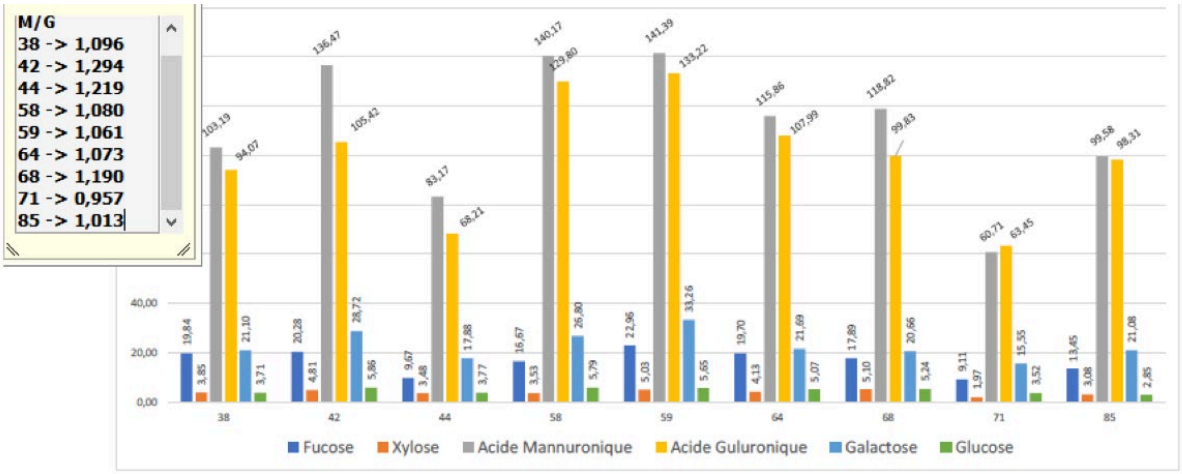

Figure 7. Monosaccharide composition of cell wall extracts of nine outlier individuals. Inset indicates the mannuronic acid to guluronic acid ratio.

Additional AIRs were stored and/or sequential extracted to carry out Fourier transform infrared (FTIR) spectroscopy analysis of cell wall material and to carry out phlorotannin assays (Figure 6).

The AIR supernatants of the 40°C 70% ethanol extractions were retained for phlorotannin assays but these assays did not produce usable results (low concentrations and low variance, although it is also possible that phlorotannins were degraded during storage of the samples). Otherwise, all the other supernatants were discarded and just the insoluble residue (alcohol insoluble residue, AIR) was retained for monosaccharide and FTIR assays.

The AIR was then extracted with 2% $CaCl_2$ at 70°C which precipitates most alginates. The supernatant corresponded to the fucan fraction. The residue was then extracted with 3% $NaHCO_3$ at 80°C to solubilise alginates. The supernatant from this extraction corresponded to the alginate fraction.

A first series of FTIR analyses were carried out on the nine "outlier" individuals (see above). Based on the results of this preliminary analysis, we decided to not carry out the FCSP analysis for the whole F2 family (due to low variation).

A full set of FTIR spectra was then generated for 121 F2 individuals: 6 spectra for the AIR fractions (2 acquisitions for each of 3 AIR fractions) and 6 spectra for the alginate fractions (for

a total of 1452 spectra). Analysis of the data indicated that there were differences for several individuals based on both the AIR and the alginate extracts. The AIR samples indicated two clear subpopulations plus one outlier individual. The PCA was less discriminant for the alginate samples and there also appeared to be an effect of date of extraction so this needs to be taken into account when comparing individuals. As the AIR is more representative of the whole cell wall content which also includes alginates, with less apparent environmental (date of extraction) variations, we focused on this fraction only. The FTIR spectra were analysed to extract numerical values from 4000 to 500 cm-1 and that could be used as phenotypic parameters for QTL analysis. A total of 95 spectral peaks appeared to exhibit variance between individuals and data for these wavelengths were extracted for QTL analysis.

Genotyping

The ddRAD-seq method was used to genotype 192 individuals including the two grand-parents (strains SLPER139 and SLSTG055), a single F1 individual (strain F1.27.2) and 189 diploid progeny derived from the F1 individual (these included the 129 phenotyped individuals that had been grown on the open sea rope system plus 60 additional individuals derived from the same parent sporophyte but raised in the laboratory, which were genotyped but not phenotyped). Genomic DNA was digested with *Pst*I and *Hha*I and adapters added to include indexes and barcodes before selection of 150 to 600 bp fragments for amplification and sequencing. The ddRAD library was sequenced (125 base reads) on a total of 5 lanes giving 966 million raw-reads. After filtering and trimming, 660 million reads remained (average read number per sample: 3.5 million). Mean coverage per sample was 16.

The *S. latissima* reference genome

A high quality *S. latissima* genome assembly (531 Mbp, 4592 scaffolds) generated by the Phaeoexplorer project was used as the reference scaffold for the generation of the genetic map and QTL identification. To produce the genome sequence, Illumina paired end reads and Nanopore long reads were generated from DNA extracted from haploid gametophyte cultures of the female *S. latissima* strain SLPER63f7, which is derived from a sporophyte isolated at Perharidy, Roscoff, France in 2015. The assembled genome was annotated by the Genoscope sequencing centre using published RNA-seq data generated by Cátia Monteiro (Machado Monteiro *et al.*, 2019).

Construction of the sporophyte genetic map

Five different genetic maps were initially constructed with the following characteristics:

- Map1: SNPs were called using the Stacks version1 pipeline and a denovo_map approach and the map was constructed with LepMap3 program.
- Map2: SNPs were called using the Stacks version2 pipeline and a denovo_map approach and the map was constructed with LepMap3 program.
- Map3 and map4: SNP calling with DiskoSnp-RAD (Gauthier *et al.*, 2020) with a reference_map approach using both an improved re-assembly of the *S. japonica* genome (Ye *et al.*, 2015) carried out in Roscoff (using Tassel and Stacks) and a draft (Illumina DNA-seq only) *S. latissima* female genome produced by Phaeoexplorer. Map construction with LepMap3.
- Map5: loci clustered with CD-Hit (shown to be more efficient by LaCava *et al.*, 2020) and then reads were mapped onto these putative loci to call SNPs with Stacks v2. Map constructed with LepMap3.

The construction of the genetic map was the repeated using the PacBio-based assembly of the genome of the female *S. latissima* strain as the reference. The final map had 4266 markers on 31 linkage groups. This genetic map represents an important resource for future genetic improvement of European *S. latissima*.

QTL analysis

QTL mapping was carried out with the R qtl and qtl2 packages using the phenotypic data indicated in Table 1.

Table 1. Phenotype data used to analyse for the presence of QTLs

Phenotype	**Explanation**	**Analysis**
Longueur_total	Length of individual without crampon	Morphometry
Longueur_stipe	Length of the stipe	Morphometry
Largeur	Width of blade at widest point	Morphometry
Longueur_sans_epiphyte	Length of the part of the blade without epiphytes	Morphometry
Longueur_peu_epiphytes	Length of the part of the blade with few epiphytes	Morphometry
Longueur_bcp_epiphyte	Length of the part of the blade with many epiphytes	Morphometry
FvFmmoy(21°C-7j)	Average of Fv/Fm values for triplicate disks treated as follows: 4 days acclimatisation at 13°c followed by 7 days at 21°C (moy(21°C-7j)).	Stress (Fv/Fm)

FvFmmoy(23°C-48h)	Average of Fv/Fm values for triplicate disks treated as follows: 4 days acclimatisation at 13°c followed by 2 days at 23°C (moy(23°C-48h))	Stress (Fv/Fm)
FvFmmoy(13°C-CTRL)	Average of Fv/Fm values for triplicate disks treated as follows: 4 days acclimatisation at 13°c followed by 7 additional days at 13°C (moy(13°C-CTRL))	Stress (Fv/Fm)
FvFmmoy(RECUP)	Average of Fv/Fm values for triplicate disks treated as follows: 4 days acclimatisation at 13°c followed by 7 days at 21°C and then 14 days at 13°C (moy(RECUP))	Stress (Fv/Fm)
FvFm_état_21°C_ en%duCTRL	Photosynthetic state of 21°C stressed as percentage of photosynthetic state of control (moy(21°C-7j) / moy(13°C-CTRL) expressed as a percentage).	Stress (Fv/Fm)
FvFm_état_après_récup_ en%duCTRL	Photosynthetic state after 21°C stress and recovery as percentage of photosynthetic state of control (moy(RECUP) / moy(13°C-CTRL) expressed as a percentage).	Stress (Fv/Fm)
FvFm_état_après_récup_ en%duStress_21°C	Photosynthetic state after 21°C stress and recovery as percentage of photosynthetic after 21°C stress (moy(RECUP) / moy(21°C-7j) expressed as a percentage).	Stress (Fv/Fm)
FvFm_état_23°C_ en%duCTRL	Photosynthetic state of 23°C stressed as percentage of photosynthetic state of control. moy(23°C-48h) / moy(13°C-CTRL) expressed as a percentage	Stress (Fv/Fm)
Note_stress	Stress score (out of 5) based on Fv/Fm measurements (low score = high sensitivity to stress).	Stress (Fv/Fm)
Note_récup	Recuperation score (out of 5) based on Fv/Fm measurements (low score = decrease in photosynthetic state during recuperation).	Stress (Fv/Fm)
Valeur_FvFm	Sum of the Note_stress and Note_récup (low score = high sensitivity to stress and/or low capacity to recover).	Stress (Fv/Fm)
Valeur_cond(x2)	Stress score based on conductivity measurements (low score = high sensitivity to stress). Scored out of 5 and then multiplied by two to be comparable with Valeur-Fv/Fm.	Stress (conductivity)
Electrolyte_loss	Electrolyte loss due to stress as % of total electrolytes	Stress (conductivity)
Disque1	Weight of disk 1	Growth
Disque2	Weight of disk 2	Growth
Disque3	Weight of disk 3	Growth
max_weight	Weight of the heaviest disk of the 3	Growth
DryWt/FreshWt_%	Dry weight as percent of fresh weight	Growth
protein	Protein contant	Metabolism
AA	Amino acid content	Metabolism

NO3	Nitrate content	Metabolism
NH4	Ammonium content	Metabolism
FTIR_WL x 95	Peak values at 95 wavelengths that showed variability across individuals. data was treated as raw, normalised, detrend and smoothed	Cell wall

The analysis therefore used 28 "classic" phenotypic expressions (morphometry, stress, growth, metabolism) plus 95 FTIR-based cell wall phenotypes (i.e. wavelengths; treated as raw, normalised, detrend and smoothed data), 123 phenotypic parameters in all.

Searches for QTLs were carried out using classic interval mapping and using a genome scan based on a linear mixed model which took into account kinship. For the "classic" phenotypes, up to five QTLs were identified depending on the approach used. For the FTIR data, two QTLs were detected at 83-86 cM on LG3 and at 23 cM on LG1. The QTLs corresponded to wavelengths of 890 and 905 corresponding to ß-glucanes and perhaps also mannuronic acid.

Conclusion for the sporophyte family QTL analysis

The analysis of the segregating sporophyte family has detected QTLs for both "classic" phenotypes and for cell wall characteristics based on FTIR analysis. However, overall, analysis of the data indicated that there was a limited power to detect QTLs in this family. This may have been due to several factors including the quality of the maps, the size of the F2 family, the level of genetic variance (both grandparents were from Brittany, although from the distinct northern and southern zones) or the choice of phenotypes. Nevertheless, QTLs that have been identified will serve as a starting point for future breeding efforts and for searches for candidate genomic regions associated with aquanomically important characteristics. QTLs linked to stress resistance will be relevant to future efforts to understand how seaweed populations and crops will respond to climate change. The FTIR -based analysis of cell wall components represents a particularly interesting approach to develop high-throughput methodologies for phenotyping important cell wall biomolecules and for breeding initiatives aimed at increasing and improving the content of these molecules.

Establishment of a segregating F2 gametophyte family

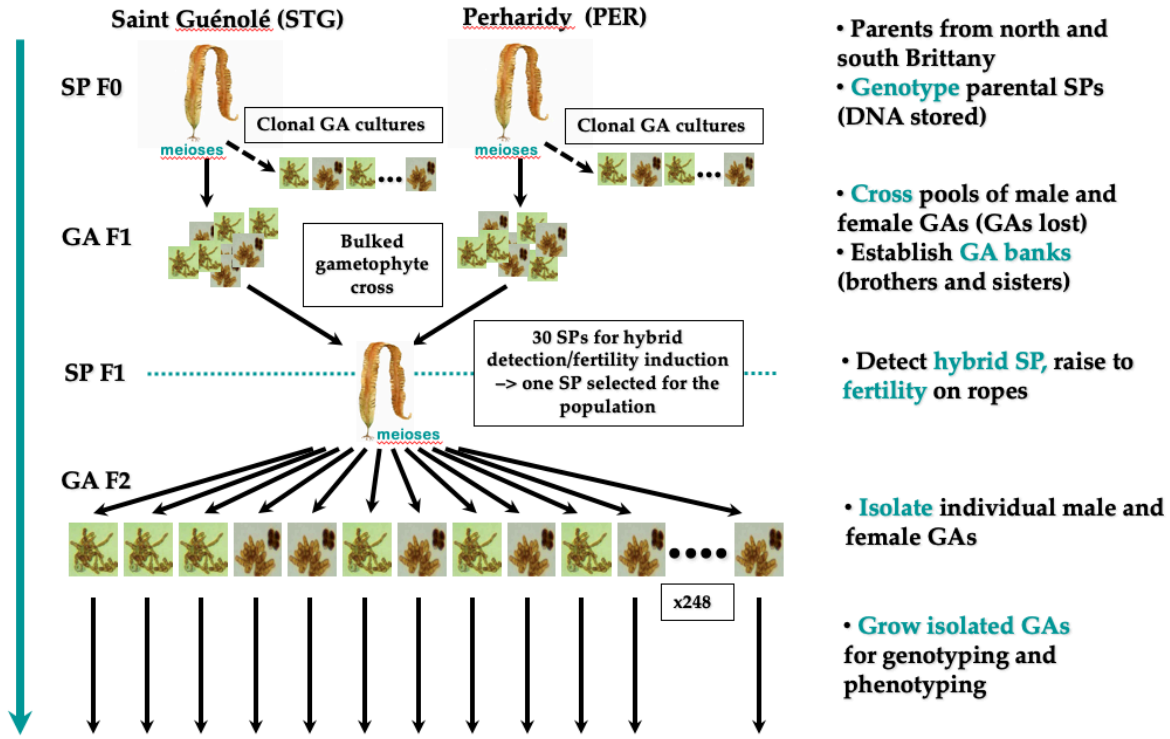

Figure 8. Breeding strategy for the generation of a segregating family of *S. latissima* gametophytes for QTL mapping.

The gametophyte QTL family was derived from the same SLPER139 x SLSTG055 cross as was used to generate the sporophyte family (Figure 8). As described above, mixes of male and female gametophytes, derived by sporulation of each the two strains, were combined in a bulk gametophyte cross (February 2016). The F1 sporophyte progeny from this cross were then cultivated on an open sea rope system near Roscoff in the Morlaix bay (from April 2016) and the F1 sporophytes were genotyped using microsatellite markers in order to select a hybrid F1 individual (F1.27.2).

The hybrid F1 individual (strain F1.27.2) was sporulated to produce a mix of male and female gametophytes[2]. To create the F2 gametophyte family, 248 gametophytes (156 males, 92 females[3]) were isolated as individual cultures in March and April 2017.

[2] These gametophytes were also bulk crossed to create the F2 sporophyte QTL family (see earlier section).
[3] The individuals can be difficult to sex based on morphology, especially if they are stressed (causing cell expansion).

A copy of the family was sent from SBR to SAMS. At SAMS the gametophytes are grown under red light 12h:12h at 13°C, in sterile, filtered Provasoli-enriched (10ml/L; https://www.ccap.ac.uk/media/documents/Modified_Provasoli.pdf) seawater (PES). The gametophytes have been propagated every 3 to 4 months by fragmentation and renewal of the medium.

Planning and preparation of the phenotyping experiment

The phenotype measured for the gametophyte family was resistance to temperature stress. Before carrying out the phenotyping of the entire family, a test experiment was first carried out with eight of the progeny strains to validate the propose experimental protocol. This experiment involved an acclimation period of 2 days, stress at 23°C for 3 days and recovery for 8 days. Fv/Fm measurements were used as a proxy to measure the stress state of the individuals. Measurements were made at the ends of the acclimation, stress and recovery periods.

Before the acclimation period of the experiment, the fragmented filaments of eight test strains (>40 µm filaments) were grown in PES under white light (20 µE.m-2.s-1, 12h:12h) at 15°C in 140 mm Petri dishes. Monitoring of Fv/Fm during pre-acclimation indicated that only strains with Fv/Fm >0.485 (the minimum value observed during the stabilisation) on the day before the start of the experiment (i.e. day -1) should be retained for phenotyping. For some strains, at least 9 days of pre-acclimation were necessary to stabilise the Fv/Fm values (Figure 9).

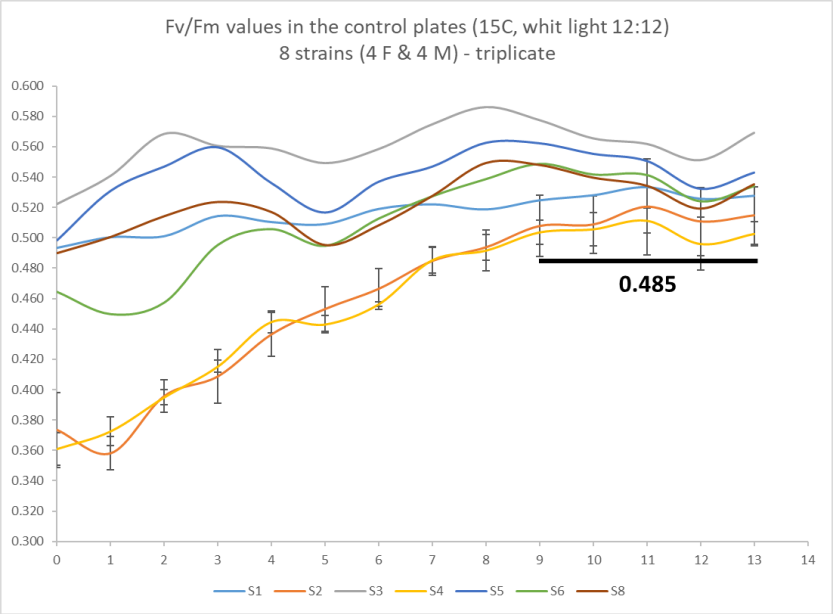

Figure 9. Evolution of Fv/Fm values over 14 days for 8 different *S. latissima* gametophyte strains. Some of the strains required 9 days before the Fv/Fm values reach a stable state (Fv/Fm = 0.485).

The strains with Fv/Fm >0.485 (six strains) were transferred into a 24 well glass bottom plate (Greiner Bio-One, SensoPlate™, ref: 662892) in 2 ml fresh PES per well and maintained under the same conditions (white light, 20 µE.m-2.s-1, 12h:12h, 15°C) for 2 days. Fv/Fm values were measured at the beginning (day 0) and at the end (day 2) of this acclimation period.

At day 2, after carrying out the control Fm/Fv readings, the plates were transferred into another incubator under the same conditions (white light, 20 µE.m-2.s-1, 12h:12h) but at 23°C (the incubator was set at 23°C but the temperature increased to 24.5°C during the day) for 3 days. The stress Fv/Fm values were measured at the end of the stress period (day 5). Analysis of this preliminary experience results showed that the impact on Fm/Fv was maximal at 3 days (Figure 10).

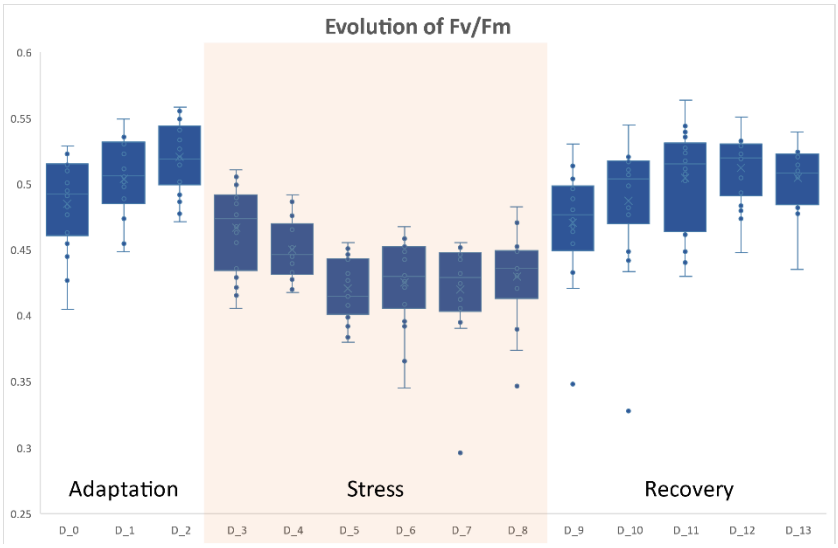

Figure 10. Evolution of Fv/Fm during adaptation (days 0 to 2), temperature stress at 23°C (days 2 to 8) and recovery (days 8 to 13). The impact on Fm/Fv was maximal at 3 days.

At day 5 the plates were transferred back to the control conditions (white light, 20 µE.m-2.s-1, 12h:12h, 15°C). Analysis of this preliminary experience showed that strains started to recover after 4 days (averages and standard-deviations were stable at this time).

Differences were observed between the responses of the six strains in this preliminary experiment, which was encouraging (Figure 11).

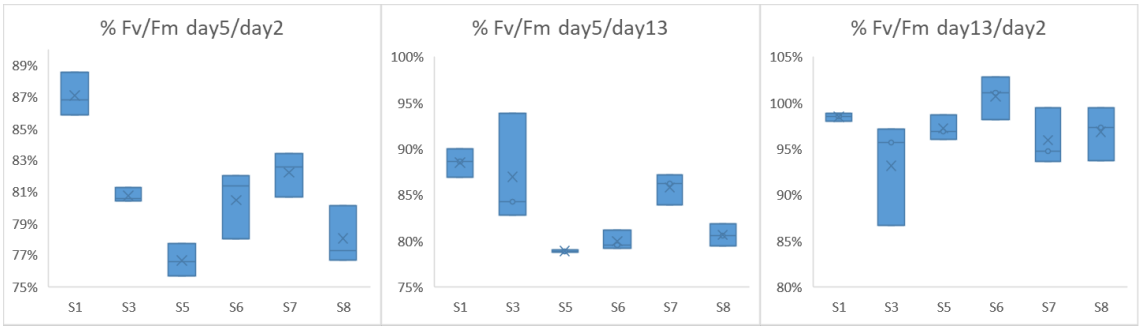

Figure 11. Results of the preliminary experiment showing the % Fv/Fm Sress/Control (day 5/day 2), % Fv/Fm Stress/Recovery (day 5/day 13) and % Fv/Fm Recovery/Control (day 13/day 2 data). The six *S. latissima* strains tested exhibited different responses.

Phenotyping for resistance to temperature stress

Based on the preliminary temperature stress experiment, a protocol was established for the full experiment based on a stress at 23° for three days (Figure 12).

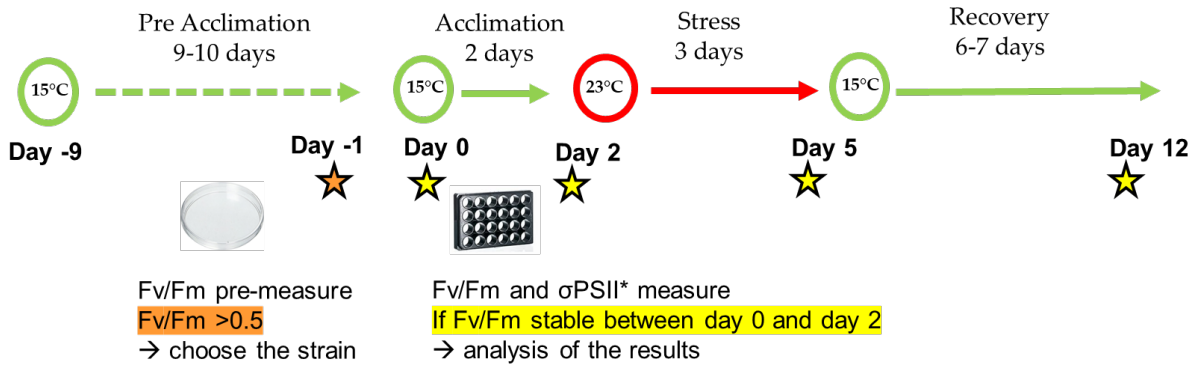

Figure 12. General overview of the phenotyping protocol used to evaluate the temperature stress resistance of the *S. latissima* gametophyte segregating family.

The protocol involved measurement of the following phenotypic parameters:

1. Average Fv/Fm at day 2 (Control)
2. Average Fv/Fm at day 5 (Stress)
3. Average Fv/Fm at day 12 (Recovery)
4. % Stress/Control (day 5/day 2)
5. % Stress/Recovery (day 5/day 12)
6. % Recovery/Control (day 12/day 2)
7. Note stress i.e. category [0-5] based on % Stress/Control
8. Note recovery i.e. category [0-5] based on % Stress/Recovery
9. Note FvFm (Note Stress + Note Recovery)
10. Average Fv/Fm at day 0
11. % day 0/Control (to retain only strains with stable Fv/Fm)

Analyses used at least 4 replicates per F2 individual (4 replicates * 179 individuals = 716 samples / 24 wells = 30 plates / 4 = 8 batches, although in reality 12 batches were actually analysed) with pseudo-random distribution of the strains in each plate.

A total of 179 progeny (97 males and 82 females) were selected from the 248 clonal gametophytes based on the following criteria:

- Strains that had survived in both the SBR and SAMS
- Strains with sufficient biomass for genotyping (SBR) and phenotyping (SAMS)

Phenotype data has been obtained for all but three of these strains (96 males and 80 females in total) and is currently being analysed. In particular, we have noted that some of the data sets (replicated 24-well plates) have particularly low Fv/Fm ratios (Figure 13). We are currently investigating the source of this variance and testing different approaches that might be used to address the problem, including elimination of strains with abnormal or unstable Fv/Fm values and normalisation approaches.

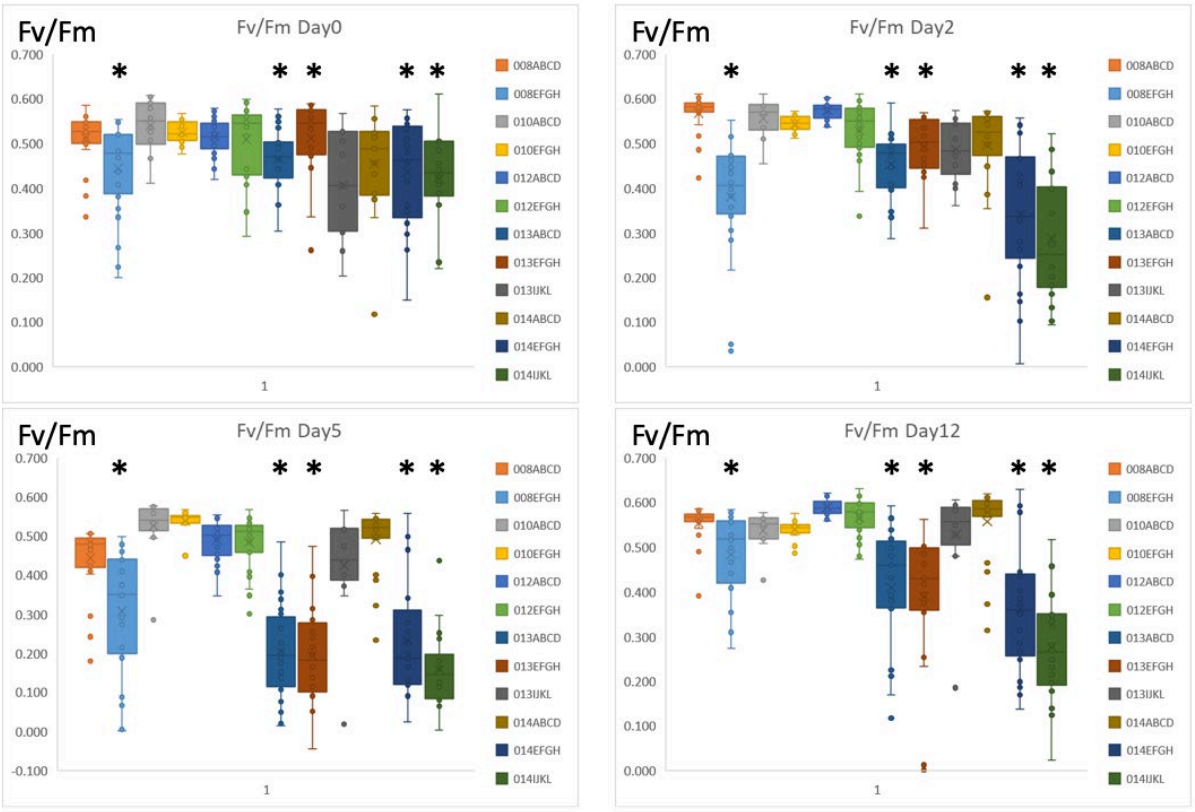

Figure 13. Fv/Fm measurements carried out during the temperature stress phenotyping experiment. Each boxplot represents 4 replicates of 24 strains. Asterisks indicate sample sets with outlier distributions ((quartile 1 - quartile 3)x1.5.).

Genotyping

The 97 males and 82 females of the gametophyte family are being genotyped using the ddRAD-seq method. DNA has been extracted for all 179 strains and ddRAD libraries are being constructed for sequencing.

Generation of a genetic map and QTL analysis

The ddRAD-seq data will be analysed using the same approach as was used for the sporophyte family to generate a gametophyte-based genetic map. This map will then be compared with the map obtained using the sporophyte family as a means to assess the robustness of the two maps and to ascertain whether there are any differences of interest.

The phenotype data from the gametophyte temperature stress experiment will then be analysed with the aim of identifying QTLs for stress resistance. It will be particularly interesting to compare the results obtained with the sporophyte and gametophyte families, in particular to determine if there is a correlation between the stress tolerances of the two generations. For example, do sporophytes and gametophytes that are resistant (or sensitive) to high temperature share genetic variants? Similarly, do the analyses of the sporophyte and gametophyte families for temperature sensitivity QTLs identify overlapping sets of loci in the two life cycle generations?

Evaluation of effects of temperature stress on the sex ratio

Shifts in sex ratio in response to sub-optimal temperatures have been described in several kelp species (Izquierdo *et al.*, 2002; Nelson, 2005; Oppliger *et al.*, 2011). At high temperatures (between 17 and 20°C) *S. latissima* has been reported to produce more male than female gametophytes (Lee and Brinkhuis, 1988). In this study, female gametophytes grew at 20°C, but with a lower rate than between 4 and 17°C and their fecundity was repressed (Figure 14). A similar bias was observed in a more recent study (Monteiro *et al.*, 2019).

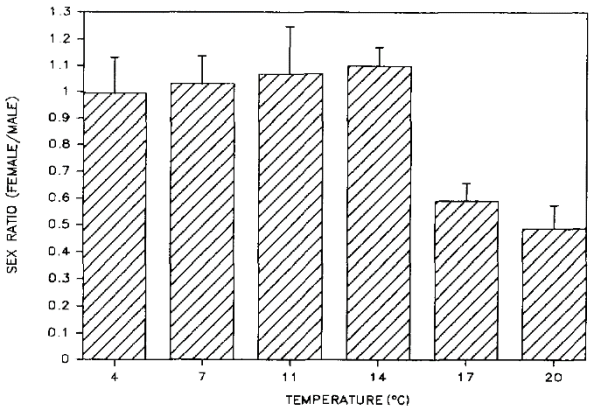

FIG. 5. Sex ratio of *L. saccharina* vs. temperature with standard error bars. Sex ratio was determined as female gametophytes/male gametophytes within the culture three weeks after meiospore inoculation.

Figure 14. Reproduction of a figure from Lee and Brinkhuis (1988) showing temperature-dependent changes in the gametophyte sex ratio.

Analysis of the gametophyte family will also look at differential effects of temperature stress on male and female individuals. In addition, sex-specific effects will have to be taken into account during the analysis as these earlier studies indicate that the sex-determining region of the sex chromosome may have an important effect on temperature stress resistance through its role in sex-determination.

Conclusion for the gametophyte family QTL analysis

We have not been able to fully complete the analysis of the gametophyte segregating family within the timeframe of the Genialg project but the majority of the work has been carried out for this part of the project: the segregating family has been isolated and cultured, the family has been phenotyped and the genotyping is almost complete. We expect to have completed the analysis of the gametophyte family within the next three months.

Genome-wide association study of morphometric and biochemical characteristics in European *Saccharina latissima*

The objective of this study was to apply a GWAS approach (Atwell *et al.*, 2010; Rafalski, 2010) to a Europe-wide collection of *Saccharina latissima* isolates to identify loci that influence morphometric and biochemical parameters.

Overall organisation of the experimentation

The GWAS experiment was carried out in two rounds (Experiment 1 in 2018 and Experiment 2 in 2019). Experiment 1 analysed individuals from 10 localities and Experiment 2 individuals from 13 localities. In total, the two experiments analysed individuals from 21 localities (2 localities were analysed in both experiments).

Europe-wide sampling of *S. latissima* populations

Parental sporophytes were from 21 localities (population codes: TRO, KOS, GOT, HEL, EYE, POR, SHI, ATB, TRA, VEN, PTW, AUD, SMA, PER, PMI, CON (initially called PDC), LOC, PIR, NOR, ESP, AMO) for the two GWAS experiments (Figure 15, Table 2).

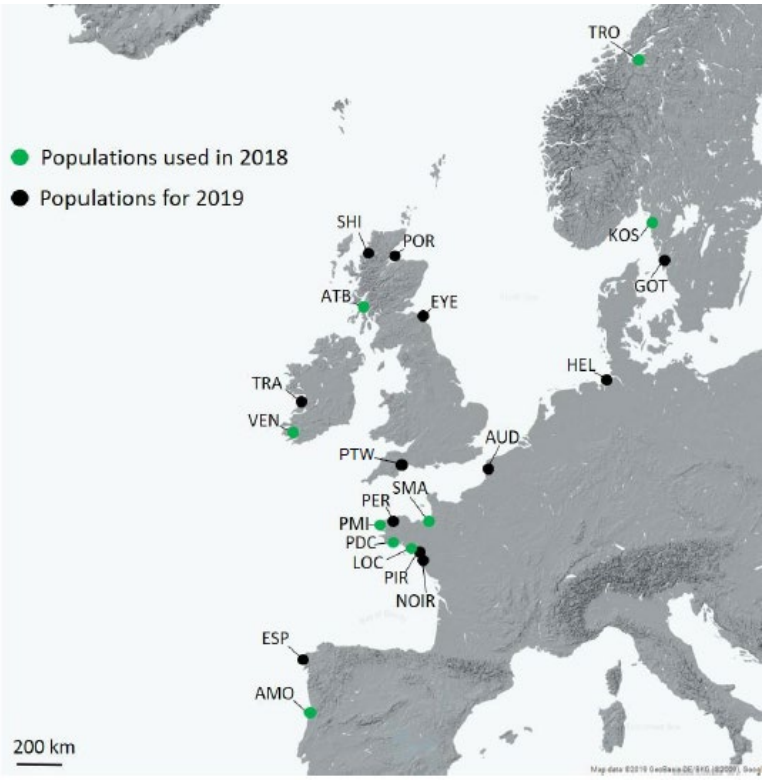

Figure 15. *S. latissima* sampling sites

Table 2. Populations sampled for *Saccharina latissima* sporophytes.

Population code	Ocean or sea	Country	Region	Location	Site	Latitude	Longitude	Date of collection	N° indiv. sampled
GWAS1									
VEN	Celtic Sea	Ireland	County Kerry	Dingle Peninsula	Ventry bay	52.11575	-10.36756	20/02/2018	30
TRO	Norwegian Sea	Norway	Sør-Trøndelag	Trondheim	Ringvebukta	63.45261	10.46119	04/02/2018	30
KOS	North Sea	Suède	Västra Götaland	Tjärnö*	Kosterhavet national park	58.83552	10.99055	27/01/2018	30
PDC > CON	NE Atlantic	France	Brittany	Concarneau	Pointe du Cabellou	47.85972	-3.91638	20/12/2017	30
LOC	NE Atlantic	France	Brittany	Locmariaquer	Pointe de Kerpenhir	47.55705	-2.92567	04/12/2017	30
PER	NE Atlantic	France	Brittany	Roscoff	Perharidy	48.73087	-4.00453	05/12/2017	30
SMA	NE Atlantic	France	Brittany	Saint-Malo	Saint-Malo	48.65245	-2.03801	05/12/2017	30
PMI	NE Atlantic	France	Brittany	Lanildut	Parc Marin d'Iroise	-4.79261	48.47902	22/01/2017	30
ATB	NE Atlantic	Scotland	Argyll and Bute	Oban	Atlantic Bridge	56.31620	-5.58350	15/12/2017	30
AMO	Atlantic Ocean	Portugal	Norte e Porto	Viana do castelo	Amorosa	41.64187	-8.82340	03/02/2018	30
GWAS2									
ESP	Atlantic Ocean	Spain	Uia	Ría de Muros y Noya	Punta Polveira	42.78667	-8.96611	20/02/2019	19
TRA	Celtic Sea	Ireland	County Clare	New Quay	Tra Li	53.15625	-9.07312	20/02/2019	21
PIR	NE Atlantic	France	Pays de la Loire	Vendee	Piriac-sur-mer	47.38189	-2.53866	06/12/2018	20
PTW	NE Atlantic	England	Southwest	Cornwall	Porthallow	-50.06919	-5.08119	20/03/2019	10
LOC	NE Atlantic	France	Bretagne	Locmariaquer	Pointe de Kerpenhir	47.55705	-2.92567	21/04/2019	9
POR	North Sea	Scotland	Highlands	Tarbat Peninsula	Portmahomack	57.83290	-3.84350	24/01/2019	25
AUD	NE Atlantic	France	Pas-de-Calais	Cape de Gris Nez	Audreselles	50.83269	1.58602	21/02/2019	22
PER	NE Atlantic	France	Brittany	Roscoff	Perharidy	48.73087	-4.00453	21/02/2019	30

SHI	NE Atlantic	Scotland	Highlands	Shieldaig	Shieldaig	57.52580	-5.65000	06/12/2018	23
EYE	North Sea	Scotland	Berwickshire	Eyemouth	Eyemouth	55.83990	-2.06190	23/01/2019	26
GOT	North Sea	Sweden	Bohuslän	Mollösund	Mollösund	58.06367	11.47574	07/02/2019	25
NOR	NE Atlantic	France	Loire-Atlantique	Noirmoutier	Pointe du Devin	46.99128	-2.30273	22/01/2019	17
HEL	North Sea	Germany	Helgoland	Helgoland	Helgoland Kurpromenade beach	54.18180	7.89155	21/03/2019	32

The following information was noted for each sampling session: name of the site, date and hour, tide coefficient, corrected depth if subtidal, GPS coordinates of the site, water temperature, names of the operators.

Each individual was identified by an ID code that included "SL" for *Saccharina latissima*, a three-letter corresponding to the sampling site (e.g. "ELL" for Ellenabeich) and a number. For example, the third sporophyte sampled at Ellenabeich was "SLELL3".

At the time of sampling, five morphometric measurements were carried out for each sampled sporophyte (Figure 16).

ID	Width Stipe	Length Stipe	Width Blade	Length Blade	Length Thallus
SLELL1	0.61	11.0	24.0	60.0	71.0
SLELL2	0.82	41.0	27.0	102.0	143.0
SLELL3	0.64	18.0	21.5	84.0	102.0

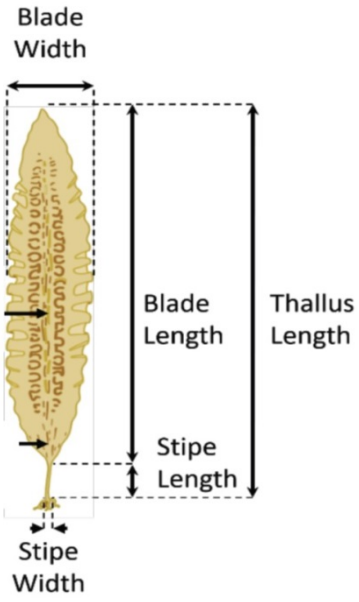

Figure 16. Morphometric parameters measured for sampled sporophytes. The top panel shows an example of morphometric data recorded for three individuals. The bottom panel indicates how the measurements were made.

Sampling involved simultaneous collection of vegetative sporophyte blade tissue for DNA extraction (2 cm^2 blade fragment from near the meristem area and the edge of the blade placed in a sachet with silica gel after rinsing with freshwater and drying with paper) and fertile blade tissue (sorus) for spore release to produce gametophyte cultures (four 1 cm^2 blade sorus fragments rinsed with freshwater, dried with a paper towel and placed in a 50 ml Falcon tube containing 45ml of sterile filtered seawater and two microscope slides to collect settled spores). The samples were then sent to SAMS (Oban, Scotland) for treatment.

Sporulation and generation of F1 sporophytes by selfing of bulk gametophytes

To induce sporulation (Figure 17), the Falcon tubes containing the four samples of blade sori for each individual were incubated at 13°C in the dark (Nielsen *et al.*, 2016; Ratcliff *et al.*, 2017). Following sporulation, the 45 ml of seawater was poured into three 10 cm Petri dishes and the two microscope slides placed in two of the Petri dishes. PES (Starr and Zeikus, 1993; Yao *et al.*, 2020) was then added and the Petri dishes placed under red light (10-40 µmoles m-2s-1, 700 nm) at 15°C to allow the gametophytes to develop (Perez *et al.*, 1992; Andersen, 2005; Arbonna and Molla, 2006; Tamigneaux *et al.*, 2011, 2013; Martins *et al.*, 2017).

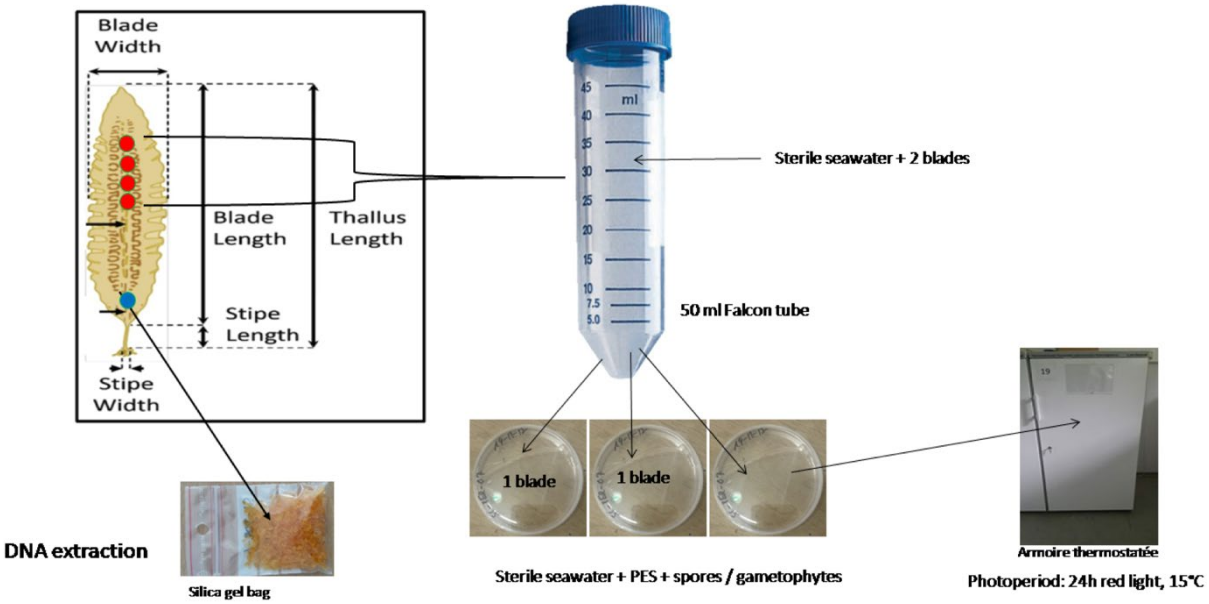

Figure 17. Sporulation of sampled sporophyte sori.

Selfing was carried out by allowing the bulked male and female gametophytes derived from a single sporophyte to cross fertilise. The three plates were examined under a binocular microscope to select the culture with the densest population of gametophytes. The microscope slide was then transferred to a 150 mm Petri dish containing PES (Starr and Zeikus, 1993; Yao *et al.*, 2020) and placed at 13°C (Fortes and Lüning, 1980; Bolton and Lüning, 1982; Andersen *et al.*, 2013; Tamigneaux *et al.*, 2013) under 20-30 µmoles m-2s-1 white light, 8h light:16h dark (Tamigneaux *et al.*, 2011, 2013). The plates were monitored under a binocular microscope and transferred to 20-100 µmoles m-2s-1 white light, 12h light:12h dark when young developing sporophytes were observed. This usually occurred about 1 month after initiation of the self-fertilisation (Figure 18). The PES was replaced with fresh medium every two weeks.

For each original sporophyte parent, 10 young sporophytes (healthy brown individuals without any developmental malformations) were transferred to two 150 mm Petri dishes (5 per plate) and allowed to grow under the same growth conditions. The PES was replaced with fresh medium every two weeks. The remaining sporophytes in the initial Petri dish were then transferred to red light (10-40 µmoles m-2s-1, 700 nm) at 15°C to inhibit growth and maintain a stock of young sporophytes.

Individual sporophytes were then selected at random for culture in 10 L carboys in aerated PES at 13°C under white daylight (OSRAM/840 neon tubes, 20-100 µmoles m-2s-1) with a 12h:12h light:dark cycle (Tamigneaux *et al.*, 2011, 2013; Yao *et al.*, 2020). The PES was renewed every two weeks. The sporophytes were cultured for between 27 and 58 days for Experiment 1 or for 30 days for Experiment 2 and harvested when they had grown to approximately 10 cm in length (young, pre-fertile thalli; Figure 18). Note that the number of sporophytes harvested was not always the same as the number inoculated due to mortality or to the presence of additional, microscopic sporophytes associating with the selected sporophytes as a "bouquet".

Figure 18. Culture of F1 sporophytes for the GWAS experiments. On the right, Petri dish with young sporophytes derived by self-fertilisation of the bulked gametophytes from a single sampled parental sporophyte. The inset shows one young sporophyte. In the middle, carboy culture system. On the right, harvested sporophytes.

GWAS experiment 1

For this experiment, each 10 L carboy contained 30 individuals from the same geographical origin. Two replicate carboys were grown for each population (20 carboys for this main experiment with two replicates plus 10 backup carboys). For the main experiment, two sporophytes were chosen at random for each selfed individual from the 10 sub-cultured sporophytes and one sporophyte placed in each replicate carboy. The experiment was carried out in three phases. The first phase with 10 carboys containing the two replicates of the Breton populations (PER, CON, PMI, SMA and LOC) was initiated with 300 individuals on the 23rd of April 2018 and harvested on either the 24th of July 2018 (5 carboys, 27 days) or the 24th of August 2018 (5 carboys, 58 days). A total of 166 individuals were harvested. The second phase with 10 carboys containing the two replicates of the populations from other European sites (KOS, AMO, ATB, TRO and VEN) was initiated with 300 individuals on the 17th May 2018

and harvested on either the 20/21/22nd of August 2018 (5 carboys, 28 days) or the 14th of September 2018 (5 carboys, 53 days). A total of 429 individuals were harvested. The 10 backup carboys, one for each population (PER, CON, PMI, SMA, LOC, KOS, AMO, ATB, TRO and VEN), were initiated with 300 individuals on the 11th of September 2018 and 261 individuals were harvested on the 11/12th October 2018 (38 days).

GWAS experiment 2

For this experiment, samples of one F1 sporophyte (selected at random from the 10 sub-cultured sporophytes) from individuals representing each of the 21 populations were mixed in the same carboy and a total of 30 carboys were inoculated in two batches (630 individuals at the start of culture, 583 individuals harvested). At the time of harvesting, individuals were assigned to their population based on genotyping with 28 microsatellite markers.

The first phase with 10 carboys was initiated with 315 individuals 8th July 2019 and harvested 271 individuals 8th August 2019 (30 days) and the second phase with 10 carboys initiated with 315 individuals 24th September 2019 and harvested 313 individuals 24th October 2019 (30 days).

For experiment 2, harvested individuals were assigned to their population of origin using microsatellite genotyping information. A panel of 40 microsatellite markers was first tested against between 20 and 27 individuals each from 13 different localities (AMO, AUD, ESP, EYE, GOT, NOI, PER, PIR, POR, PTW, SHI, TRA, VEN). This analysis indicated that a combination of 27 markers (Sacl-06 Sacl-09 Sacl-11 Sacl-37 Sacl-54 Sacl-94 Sacl-65 Sacl-90 Sacl-95 Sacl-13 Sacl-21 Sacl-32 Sacl-33 Sacl-41 Sacl-56 Sacl-60 Sacl-75 Sacl-78 Sacl-88 SLN319 SLN320 SLN34 SLN35 SLN 510 SLN 32 SLN 36 SLN 54) allowed discrimination between the different populations based on genotype. Each of the harvested individuals from experiment 2 was therefore genotyped with this panel of microsatellites and thereby assigned to their population of origin.

Phenotyping - morphometry

The harvested F1 individuals were individually photographed and the following morphometric measurements recorded:

- Stipe length (cm)
- Total length (cm)
- Blade length (cm)
- Blade width (at widest point) (cm)
- Blade surface area (cm^2)

Phenotyping - metabolic parameters (September 2020)

For both GWAS experiments, sporophyte blade tissue samples were taken after the young sporophytes had been photographed and rapidly frozen in liquid nitrogen (no more than 30 min after removal from the carboys). To carry out biochemical assays, each sample was ground to a fine powder under liquid nitrogen.

About 5 mg of tissue powder was used for each sample. All 353 samples were randomized across four different 96 well plates. For inter-plate variation, each plate contained 3-4 aliquots of an *Ulva* control sample, which was the same for all assays. Soluble metabolites were extracted with sequential hot ethanolic extraction (100%, 80% and 50% EtOH) and the insoluble pellet was resuspended in NaOH.

The metabolites assayed were nitrite, nitrate, ammonium, amino acids, proteins and four different soluble sugars. The control measurements were good in terms of absorbance per plate, *Ulva* control values for each assay and the *Ulva* controls compared with previous determinations, indicating that the measurements should be reliable.

Levels of the soluble sugars glucose, fructose and sucrose were at the detection limit and were therefore not included in the analysis. A low but probably significant amount of rhamnose was detected so this could be analysed, with caution.

Six of the measured samples had very little biomass (<0.5 mg) and may therefore be unreliable due to weighing inaccuracy.

Additional metabolites could be measured if required. The extracts are stored at -80°C and should be stable for years.

Genotyping

The F1 sporophytes were genotyped using the ddRAD-seq method. DNA was extracted from samples of 494 sporophyte individuals and two barcoded ddRAD libraries constructed for 125 bp paired-end Illumina sequencing.

GWAS analysis

The genotype and phenotype data is currently being analysed using the female *S. latissima* strain SLPER63f7 genome assembly as a reference scaffold.

Heritance analysis

To determine whether growth-related (morphometric) traits are inherited genetically, measurements for five individuals from the GWAS experiment 2 (PER276, ESP24, GOT27, HEL39, SHI15) were compared with measurements for their progeny. If there was genetic inheritance of these traits, the expectance was that variance within a sample of progeny from a single individual should have been lower than for randomly selected progeny from the five parent individuals. At least 20 progenies were analysed for each parental sporophyte (147 individuals in all). The progenies were put into culture for the same amount of time and under the same conditions as for the GWAS experiment 2 (but progeny of different parents were not mixed). Photographs were taken after the culture period and the following measurements were made: 1) stipe length, 2) total length (blade plus stipe), 3) blade length, 4) blade width 5) blade surface area. Standard deviations were calculated for each of the five groups of progeny and also for 1000 randomised datasets and a permutation test was applied to calculate the significance of the comparison between the observed standard deviations from each sample and the randomized standard deviations.

In general the expectations were fully met. The standard deviations for the progeny pools were significantly lower than for the randomised pools. The only exception was for SLESP24 but it was noted that several of the progeny of this individual were deformed so that may have affected the analysis. Overall, however, this analysis confirmed that growth characteristics are inherited, which is a fundamental requirement for the GWAS experiment.

Conclusion for the GWAS

The first major outcome of the GWAS has been the established of a large collection of gametophytes derived from 579 sporophytes sampled from 21 localities spanning a region running from Portugal in the south to Norway in the north. These gametophytes represent an important germplasm collection that is expected to play an important role in the future development of *S. latissima* breeding programs. The establishment of the phenotype and genotype datasets for this collection also represents a major advance, in itself. This information, associated with the strain collection, will provide crucial information for the planning and implementation of future breeding programs. The final step of this project, analysis of the genotyping and phenotyping data, has not yet been completed but, as all the necessary data is now available, we expect this analysis to be completed rapidly.

General conclusion

This deliverable describes the implementation of three large-scale genetic projects for the cultivated brown seaweed *S. latissima*. The sporophyte family QTL analysis has been completed and complete datasets have been collected for the two projects that are still ongoing for complementary analyses, the gametophyte family QTL analysis and the GWAS. We currently work on the two remaining projects in order to complete at least two scientific papers that will valorise these results and increase the impact of these breeding approaches. With the results of the three projects published, we will have the information necessary provide the necessary tools to develop future breeding approaches for *S. latissima* (and other cultivated kelps), to implement optimal approaches that are planned to be tested for their feasibility with seaweed farmers and to secure the resources necessary for their implementation. In that respect, the experimentation carried out for this deliverable have laid down the foundation for future efforts to breed and improve *S. latissima* cultivars and a first project is proposed within the SEAMARK consortium that is currently applying for the call HORIZON-CL6-2021-CircBio-01-09: Unlocking the potential of algae for a thriving European blue bioeconomy.

The initiatives described here and the numerous results that were obtained have also established specific resources that will be further exploited in the future. First, the material available for the

QTL families (lyophilised thallus blade disks for the sporophyte family and stock cultures for the gametophyte family) will serve to evaluate additional phenotypes with the aim of identifying genomic loci (QTLs) that influence these phenotypes. Integration of new phenotypes will be relatively easy as the genotype data and genetic maps will already have been published in a publication that is planned to be submitted as soon as some analyses are completed. In addition, as mentioned above, the strain collection established for the GWAS experiment, together with associated phenotypic (both for source sporophytes and F1 sporophytes) and genotypic data, represents and invaluable resource and biobank for future breeding efforts that will be preserve within the banking facilities of the EU infrastructure EMBRC.

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