Algal Research 55 (2021) 102280

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

A sequencing-free assay for foliose *Ulva* species identification, hybrid detection and bulk biomass characterisation

Antoine Fort [a,*], Charlene Linderhof [a], Inés Coca-Tagarro [a], Masami Inaba [a], Marcus McHale [a], Kevin Cascella [b], Philippe Potin [b], Michael D. Guiry [c], Ronan Sulpice [a]

[a] National University of Ireland Galway, Plant Systems Biology Lab, Ryan Institute, SFI MaREI Centre for Climate, Energy and Marine, School of Natural Sciences, Galway H91 TK33, Ireland
[b] CNRS, Sorbonne Université Sciences, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, CS 90074, F-29688 Roscoff, France
[c] AlgaeBase, Ryan Institute, National University of Ireland, Galway H91 TK33, Ireland

ARTICLE INFO

Keywords:
Barcoding
Cleaved Amplified Polymorphic Sequences assay
Sea lettuce
Species identification

ABSTRACT

Sea Lettuce (*Ulva* spp. Ulvophyceae, Ulvaceae) has tremendous ecological and industrial impacts, from the negative effects of green tide events to the industrial production of food, feed, and value-added products. Due to the morphological similarities between *Ulva* species, their identification requires the use of "barcoding", which relies on the sequencing of short fragments of DNA and the comparison of the obtained sequence to that of sequences present in public repositories. However, Sanger sequencing can be costly when hundreds of samples need to be analysed. In addition, "barcoding", which uses Sanger sequencing, cannot be applied directly on bulk biomass, and requires independent assessment of the individuals within, which is often not possible in commercial products. Here, we describe a novel "sequencing-free" method for species identification of foliose *Ulva* species that by-passes the drawbacks associated with Sanger sequencing. The assay uses restriction digestion enzymes which target species-specific Single Nucleotide Polymorphisms (SNPs) present within the ITS1 sequence. Digestion of the ITS1 PCR product with two enzyme mixtures allows for the discrimination of the main foliose *Ulva* species, as well as *U. compressa*, which can have a foliose morphotype. Of the species tested, only *U. pseudorotundata* and *U. arasakii* show the same digestion pattern. Since those two *Ulva* species are allopatric, we expect this issue to be of limited impact for the users. In addition to species identification, we demonstrate that the assay can be used for hybrid detection, which can have interests for *Ulva* breeding and species delimitation. Importantly, the assay can be used to detect at once the different *Ulva* species present within a bulk of *Ulva* biomass, which could allow for traceability and characterisation of the purity of *Ulva* commercial products. This study provides a new quick and cost-effective method for foliose *Ulva* species identification that could readily be extended to other species.

1. Introduction

Morphology-based identification of the common foliose *Ulva* species has proven difficult over the years. While many *Ulva* species have been shown to display subtle morphological differences [1,2], the plasticity of those morphological characteristics in response to environmental changes render species identification based solely on morphological characters uncertain. For example, some *Ulva* species can display both foliose and tubular morphotypes [3,4]. Hence, molecular identification is the method of choice for *Ulva* species determination. However, molecular identification currently relies on the sequencing of PCR products of barcode genes (typically *rbc*L, *tuf*A and ITS in *Ulva* and other algae [5–9]). Such sequencing is relatively costly and time-consuming when used on hundreds of *Ulva* strains and can also lead to erroneous identifications, as described in [10,11]. In the case of a strain-selection programme where a single foliose *Ulva* species may be desirable [12,13], the cost and labour associated with sequencing can be prohibitive. Indeed, selecting individuals belonging to a given species requires i) PCR amplification, ii) PCR products purification, iii) sequencing of PCR products and iv) species identification based on the sequencing results and comparison with database entries, which can prove challenging when misidentifications are present within the

* Corresponding author.
E-mail address: antoine.fort@nuigalway.ie (A. Fort).

https://doi.org/10.1016/j.algal.2021.102280
Received 18 October 2020; Received in revised form 26 February 2021; Accepted 5 March 2021
Available online 27 March 2021
2211-9264/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

database [14]. When considering a breeding programme with thousands of samples, a faster/cheaper approach is desirable. Moreover, traceability of seaweed based products is of great importance, and the use of Sanger sequencing does not allow for species identification within a bulk biomass containing several species at once. An approach to circumvent these issues is the detection of specific SNPs associated with a given species by Cleaved Amplified Polymorphic Sequences (CAPS) assay. CAPS assays use restriction enzymes digestion of PCR products to detect the presence (or absence) of specific SNPs in the samples. We have designed a CAPS assay that discriminates foliose *Ulva* species present in the North East Atlantic based on species-specific SNPs in the ITS1 dataset (part of the 45S rDNA genes). Such a system is particularly powerful for foliose *Ulva* samples as we previously reported a generally low sequence divergence within species, and high between-species genetic diversity [10]. Hence, individuals from the same species are likely to share the same sequence when a small DNA locus is examined, such as the ITS1. Using a set of 295 foliose strains and their associated ITS1 sequences, we have identified several species-specific SNPs that can be revealed by restriction enzymes following PCR amplification of the ITS1 locus, allowing for routine species identification without barcoding. We further describe the potential uses for such an assay, from the detection of F1 hybrids to the qualitative determination of *Ulva* species present in bulk biomass for commercial purposes.

2. Materials and methods

2.1. Foliose Ulva sample collection and DNA extraction

The samples used in this study, all foliose *Ulva* individuals of >100 cm^2 in diameter, originating from Ireland, the Netherlands, Portugal and Brittany (France) are described in [10], in addition to 185 samples from the same locations. DNA was extracted from ~5 mg of freeze dried biomass using the magnetic-beads protocol described in [15].

2.2. ITS1 dataset

All ITS1 sequences originate from [14]. Briefly, all sequences from *Ulva* species in the NCBI database were downloaded, aligned using MAFFT [16], trimmed using Trimal [17] and species delimitation performed as described by [10] using a Generalized Mixed Yule Coalescent (GMYC) model. The full alignment is available here as Supplementary File 1. After assigning a species name to each sequence and retaining only foliose species entries, the sequences were manually screened to detect species-specific SNPs, and NEB cutter online tool (http://nc2.neb.com/NEBcutter2/) used to select enzymes with restriction sites recognising the species-specific SNPs.

2.3. DNA amplification, sanger sequencing and CAPS assay

The extracted DNA was amplified using a single set of primers spanning the Internal Transcribed Spacer 1 of the 45S rDNA repeats: CAPS_ITS1F (5′-TCGTTGAACCCTCCCGTTTA-3′) and CAPS_ITS1R (5′-CGATGACTCACGGAATTCTGC-3′), *rbc*L primers SHF1 and SHR4 [18], and the *tuf*A primers from [7]. PCR amplification was performed in 25 μL reaction volume containing 1 μL of undiluted DNA, 0.5 pmol forward and reverse primers, 9.25 μL of MilliQ water and 12.5 μL of MyTaq Red mix (Bioline). The PCR programme was as follows: 95 °C for 3 min, 35 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s, and a final elongation step of 72 °C for 5 min. Amplicons were sent to LGC genomics GmbH for Sanger sequencing. Species were determined based on sequence similarity with *Ulva* species from [10,14]. NCBI accession numbers are available as Table S1. For enzymatic digestion, 5 μL of the raw PCR product was digested with the enzyme mixtures (BamHI + HpaI + BfaI +PspOMI and CviQI + BtsCI, all Fisher Scientific, and T7 endonuclease, New England Biolabs), 0.1 μL of each enzyme, 0.5 μL Tango Buffer (Fisher Scientific), and 4.4 μL H_2O. Digestion was carried out for a minimum of 4 h at 37 °C. T7 endonuclease + BamHI + CviQI + BfaI + BtsCI digestion was carried out using buffer 2 (NEB) instead of Tango buffer (Fisher Scientific). Gel electrophoresis was performed using a 2% agarose gel in TAE (Tris-Acetate-EDTA) buffer for 40 min at 100 V. The ladder used was hyperladder 50 bp (Bioline). For bulk biomass trials, the biomass (~1 kg FW) was freeze dried, and ground into a fine powder. DNA extraction and PCR assay were performed as above. For sensitivity trials, DNA from *U. armoricana* and *U. australis* was pooled in different ratios, with decreasing concentrations of *U. australis* DNA (from 50% to 1.5%), prior to PCR and restriction digestion using HpaI, BtsCI and T7 endonuclease.

3. Results and discussion

3.1. Design of a sequencing-free assay for rapid foliose Ulva species determination

Our own dataset contains sequences for 295 foliose *Ulva* strains belonging to the seven foliose species we identified during our sampling in the North East Atlantic [10], i.e. *U. armoricana*, *U. rigida*, *U. gigantea*, *U. pseudorotundata* [≡*Ulva rotundata*], *U. australis*, *U. ohnoi* and *U. fenestrata*; nomenclatural authorities follow [19]. We have renamed our previous *U. laetevirens* sequences into *U. armoricana*, given that the recently sequenced *U. laetevirens* lectotype revealed that the name is in fact synonymous to *U. australis* [20]. The clade containing all previous "*U. laetevirens*" sequences is conspecific with the sequenced holotype of *U. armoricana* [14,21,22], thus we reassigned all *U. laetevirens* sequences into *U. armoricana*.

Two hundred and seventy-two (272) out of 295 individuals produced full length ITS1 sequences. Based on these sequences, we identified restriction digestion sites containing SNPs present in all of the individuals for a given species (Fig. 1A). We identified eight SNPs containing restriction sites for six enzymes that can be used for the discrimination of foliose *Ulva* species. For example, all 100 *U. armoricana* individuals possess a unique restriction site for the enzyme BfaI in the amplified fragment of the ITS1, making it simple to discriminate *U. armoricana* amplicons from those of the 6 other species after PCR product digestion with BfaI.

The BamHI/HpaI/BfaI/PspOMI mixture discriminated *U. armoricana*, *U. rigida*, *U. ohnoi* and *U. gigantea* individuals, with the complete digestion of the ITS1 PCR product and generation of a specific band pattern for each species (Fig. 1B). *U. pseudorotundata*, *U. australis* and *U. fenestrata* samples are not digested using this mixture. For those species, another enzyme cocktail (CviQI/BtsCI) digested the ITS1 PCR product and yielded a specific band pattern that distinguished between those three species (Fig. 1B). Only *U. australis* showed a restriction site polymorphism within the 44 individuals of this species in our dataset, with the first CviQI restriction site being present in only 9 out of the 44 individuals. Nonetheless, this polymorphism creates a specific band pattern that is different from samples of the 6 other species (Fig. S1). Hence, this assay represents a sequencing-free, cheap, quick and precise method to identify common foliose *Ulva* species, particularly for the correct identification required for use as food, feed or industrial applications.

3.2. The CAPS assay allows for F1 hybrid detection

In addition to species discrimination, this assay allows for the unequivocal detection of interspecific hybrids. Hybridity is rare between foliose *Ulva* species [10], and we found a single occurrence of a F1 hybrid between *U. armoricana* and *U. rigida* within a set of 110 individuals. Using the enzymes described above, hybrids can be readily detected by the presence of intermediate band patterns indicating a mixture of two *Ulva* species in the same sample. We used our assay on the *U. armoricana* X *U. rigida* hybrid (strain U99) detected using next-generation sequencing in [10] and were able to detect it as a hybrid

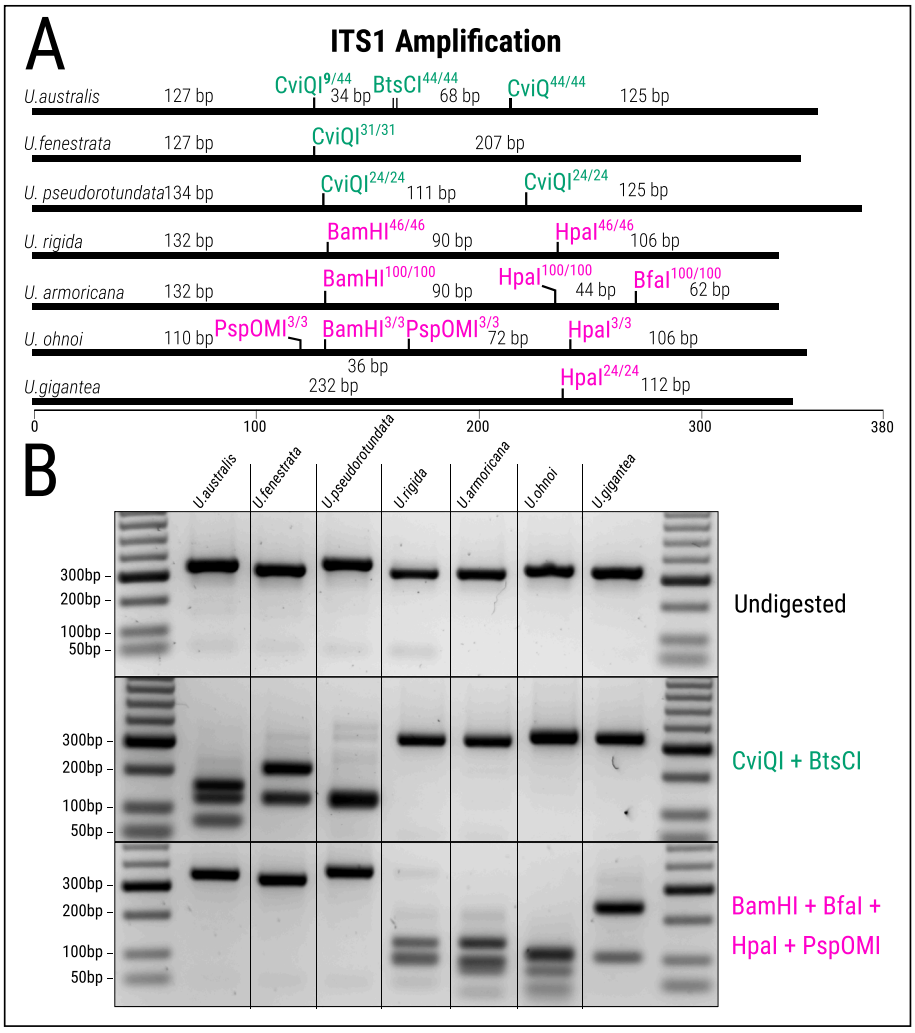

Fig. 1. Sequencing-free species determination assay for foliose *Ulva*. A) Result of the amplification of the ITS1 in each species. Restriction sites are indicated above the lines, with numbers representing the number of individuals of the species harbouring the restriction site. Expected band sizes are indicated. B) Gel results of the Cleaved Amplified Polymorphic Sequences (CAPS) assay. Top: undigested PCR amplicon of the ITS1, middle: amplicon digested with CviQI+BtsCI, bottom: amplicon digested with BamHI+BfaI+HpaI+PspOMI). *U. australis* sample does not contain the first CviQI site.

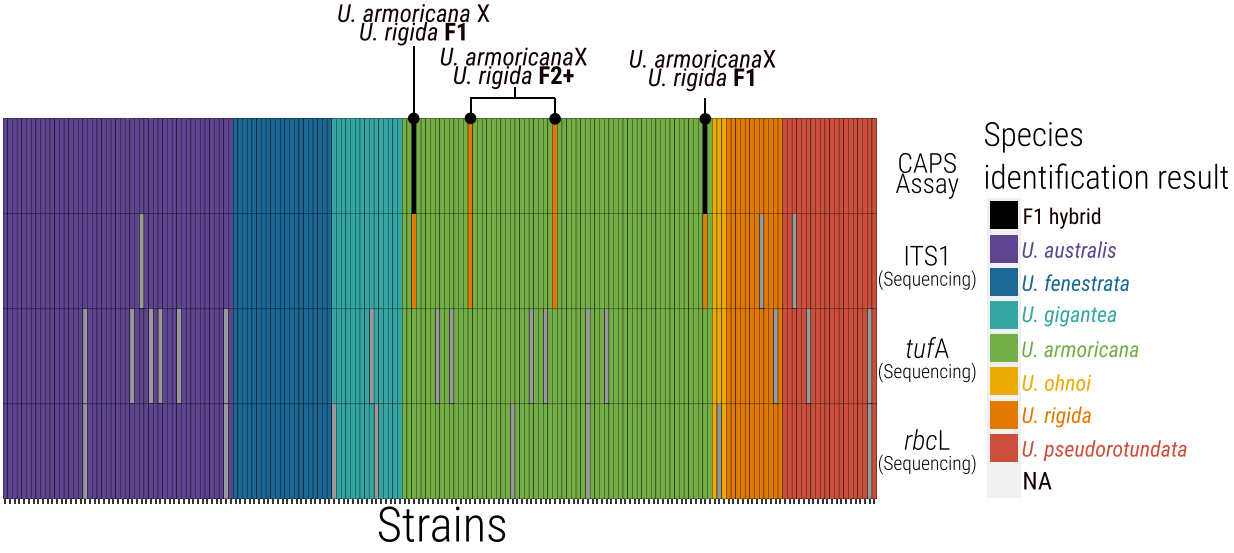

Fig. 2. Comparison of species attribution based on sequencing data and the CAPS assay. Each column represents a strain, coloured according to the species identification result based on the CAPS assay (top row) or sanger sequencing data of the ITS1, *tuf*A or *rbc*L. No sequencing trace indicates strains which failed to produce satisfactory Sanger sequencing results. F1 hybrids uncovered by the CAPS assay are indicated in black.

(Fig. S2). Indeed, digestion of the ITS1 product of strain U99 with BfaI (specific to *U. armoricana*) showed an incomplete digestion of the PCR product, indicating the presence of *U. armoricana* DNA and that of another species. When digested with BamHI (specific to *U. armoricana* and *U. rigida*), complete digestion was observed. Hence, DNA sample from strain U99 contains a mixture of *U. armoricana* and *U. rigida* nuclear DNA, which is explained by the fact that U99 is a F1 hybrid between those two species. We used the CAPS assay on 185 other foliose *Ulva* samples in addition to the hybrid U99, to investigate whether other hybrids were present. We detected one additional F1 hybrid (Fig. 2), again between *U. armoricana* and *U. rigida*.

Furthermore, we found two individuals with an apparent discrepancy in their species identity when using organellar marker genes or the ITS1 nuclear marker gene (Fig. 2), the organellar genome belonging to *U. armoricana* and the homozygous nuclear ITS1 locus belonging to *U. rigida*. These discrepancies are most likely due to previous hybridization events between the two species, which, following sexual reproduction and crossing over events, have lost the ITS1 allele from one of the original parental species. Thus, these two individuals are at least two generations older than the original hybridization event and named F2+. Interestingly, this indicates that F1 hybrids between *U. armoricana* and *U. rigida* are likely sexually competent and can generate offspring. Hence, population boundaries between the two species are unlikely to be solely determined by reproductive barriers. Those results point towards uniparental inheritance of the organellar genome in *Ulva*, since all five hybrids appear homozygous for the chloroplast barcodes, in agreement with previous reports [10,23,24].

Since the CAPS assay does not use the organellar genome, sequencing is still required for the determination of the species of the maternal parent. However, [25] have recently described an assay similar to the one presented here using structural differences in the chloroplast genome of some *Ulva* species, also described in [10] and targeting the chloroplast genome. Their assay is similarly PCR-based and allows the discrimination of *U. ohnoi*, *U. lactuca*, *U. australis* and *U. fenestrata*. In combination with the CAPS assay described here, researchers have access to a powerful screening method for foliose *Ulva* species determination and hybrid (F1 and F2+) detection.

3.3. Species determination in bulk biomass

With the rise of seaweed production worldwide for food and fodder [26], comes the potential issue of traceability of seaweed products. Indeed, seaweed pellets and seaweed in bulk biomass are difficult to characterise for the presence of different species. When a mixture of species is present within a seaweed product, barcoding cannot readily be used. In effect, amplifying a fragment such as the ITS or other barcodes from a DNA sample containing several species will not give an adequate result using Sanger sequencing, due to the presence of different DNA sequences within the same amplicon. Solutions to overcome this problem are to i) clone the PCR products and sequence each PCR product individually, or ii) use next-generation sequencing to quantify the presence of reads belonging to different species. While these solutions are working, they are relatively costly and time-consuming. Using the assay described here, it is possible to detect the presence of different foliose *Ulva* species within seaweed bulk biomass. Using a combination of the enzymes of the CAPS assay, a "fingerprint" of the species contained within the samples can be obtained. We tested the assay on seven samples of seaweed biomass from green-tide areas in Brittany (four samples taken near Roscoff [ROS], and three close to Saint Brieuc [StB], Fig. 3). Two bands were visible following amplification of the ITS1 in four of the seven samples, potentially indicating the presence of more than one species. We then digested the PCR product with a sequential combination of enzymes (see Fig. 3): CviQI/BtsCI, specific to *U. australis*, *U. fenestrata* and *U. pseudorotundata*, BfaI, specific to *U. armoricana*; BamHI, specific to *U. armoricana*, *U. rigida* and *U. ohnoi*; PspOMI, specific to *U. ohnoi*.

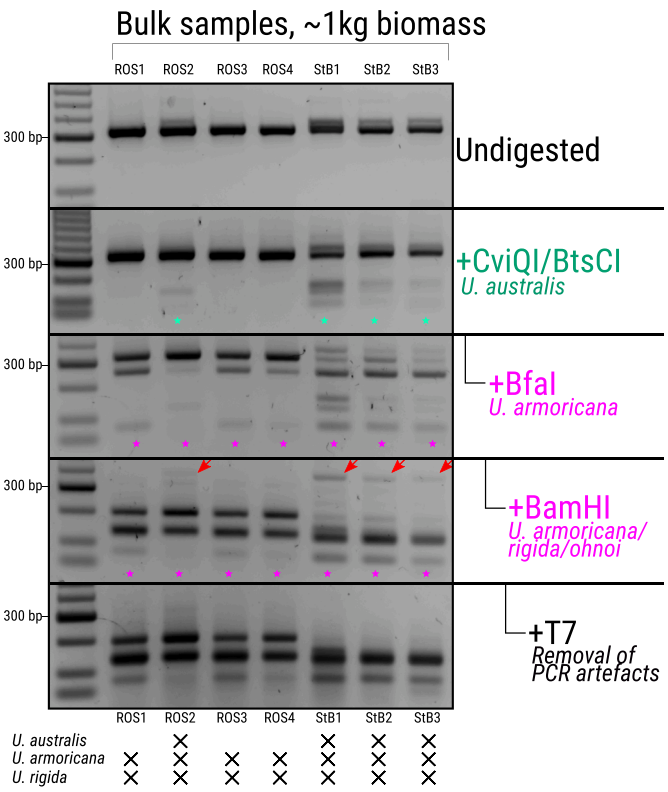

Fig. 3. *Ulva* bulk biomass analysis using the CAPS assay. ITS1 PCR amplicons and digestion with the addition of different enzymes, demonstrating the presence of multiple species. Stars indicate samples digested by the enzyme.

Digestion with CviQI/BtsCI revealed the presence of *U. australis* in four of the seven bulk samples (ROS2, containing the double CviQI *U. australis* haplotype, and StB1, 2 and 3 samples, containing the single *U. australis* CviQI haplotype). Digestion using CviQI/BtsCI/BfaI then revealed that all seven samples contained *U. armoricana*, with a leftover undigested band probably indicating the presence of *U. rigida*. This was confirmed by further digesting with BamHI restriction enzyme, which lead to the complete digestion of the PCR product in all samples. Digestion with PspOMI did not cleave the PCR product (Fig. S3), indicating that the leftover DNA band undigested by CviQI/BtsCI/BfaI indeed belongs to *U. rigida*.

However, faint leftover bands remained in four samples (Fig. 3, arrows). This band could indicate the presence of another species which does not belong to foliose *Ulva*, but it could also be from a PCR artefact. Indeed, when multiple-template PCRs are used, heteroduplex formation of PCR products can occur [27], leading to a final PCR amplicon effectively containing the amplicon from one species in the plus strand, and that of another species in the minus strand. Those amplicons cannot be cut with classical restriction enzymes such as the ones used here since they require both strands at the restriction site to be complementary. We confirmed that this additional band is a PCR artefact by performing a PCR on a mixture of *U. armoricana* and *U. australis* DNA, which indeed led to the formation of heteroduplexes, and an HpaI + CviQI (which should cleave ITS1 PCR products of all foliose *Ulva*) resistant band. This does not occur when PCR products from both species are mixed and then digested (Fig. S3). Removal of PCR artefacts such as heteroduplexes can be readily achieved using a T7 endonuclease, which will cut PCR amplicons containing mismatches between the two strands. Adding a T7 endonuclease to the final digestion with CviQI/BtsCI/BfaI/BamHI effectively removed the extraneous band (Fig. 3), confirming that all seven bulk samples only contain DNA from *U. australis*, *U. rigida* and *U. armoricana*. Hence, in order to remove the heteroduplexes formed during PCRs on DNA containing several species, we strongly recommend

the use of the T7 endonuclease at the same time as the restriction enzyme digestion. It will avoid the detection of restriction-resistant amplicons due to PCR artefacts and a remaining band will indicate the presence of another alga or protist.

While this assay is not quantitative because it relies on the amplification of the ITS1, itself part of the 45S rDNA repeats which can vary in number between species [28,29], it does provide evidence for the presence of different *Ulva* species and other possible contaminants within bulk biomass. Regarding sensitivity, we used a synthetic bulk sample containing a mixture of two species (*U. armoricana* and *U. australis*), with a dynamic range of 50% to 1.5% DNA originating from *U. australis*. The assay could detect the presence of *U. australis* in the synthetic bulk sample when its abundance was in the range of 50 to 6.25% of the total DNA, by the presence of an undigested band following *Hpa*I digestion and complete digestion using HpaI/BtsCI (Fig. 4). The band corresponding to *U. australis* disappeared when below 6.25% of total DNA, indicating that the assay has a minimum sensitivity threshold between 3.12 and 6.25%. A similar detection threshold would require the sequencing of >30 clones of PCR amplicons. For higher sensitivity, Next-Generation sequencing would be required, but the cost and time associated to such methodology can be prohibitive. Overall, our enzymatic assay could be used for traceability and detection of the purity of *Ulva* products, and this method could be extended to other commercial macroalgal species.

3.4. Robustness of the CAPS assay based on ITS1 sequences in the literature

We investigated the robustness of the assay by mining an ITS1 dataset containing all *Ulva* ITS1 vouchers in the NCBI database (in addition to those of this study, see [14]) for the presence of restriction sites for the 6 enzymes. We restricted our comparison to distromatic foliose *Ulva* species, which can readily be distinguished from monostromatic tubular *Ulva* species prior to DNA analysis. We however added the normally monostromatic and tubular *U. compressa* [≡ *Enteromorpha compressa*], as this species can occasionally display a foliose morphotype [30]. It should be noted that this analysis only counts a restriction site as present or absent if the sequence does not possess gaps/undetermined nucleotide at those positions. Of the ~40 species present in the ITS1 NCBI dataset, 11 belong to distromatic foliose species, of which seven were present in our study, in addition to *U. compressa*. The assay appears very robust for 10 of the 12 species (Fig. 5): all of *U. armoricana*, *U. australis*, *U. fenestrata*, *U. ohiohilulu* and *U. expansa* individuals harbour the same discriminating restriction sites, and only minor discrepancies were found in *U. rigida* (1/47 sequences), *U. lactuca* (4/25 sequences), *U. ohnoi* (1/29 sequences) and *U. compressa* (1/85 sequences). The presence of rare allelic variants in individuals of those species could be genetically true but could also be explained by errors during sequencing, or the presence of F1 hybrids/contaminating DNA, which we cannot assess without access to the chromatogram trace or repeating the Sanger sequencing on those entries. The only case where the assay cannot discriminate between distromatic foliose species is between *U. pseudorotundata* and *U. arasakii*, which both contain the same restriction sites for CviQI, despite considerable genetic variation between the two species (Fig. 5). However, *U. arasakii* [31] is currently known only from the Sea of Japan, while *U. pseudorotundata* is only known from Ireland, Brittany and Portugal, an inability to distinguish between them using this assay is not an issue since both species are unlikely to be present in the same areas.

3.5. Limitations of the methodology

Our methodology is aimed at performing species identification of single samples and bulk biomass of foliose *Ulva* species. Outside of the possible presence of rare allelic variants potentially producing erroneous species identification (Fig. 5), the assay is well suited to single foliose *Ulva* sample species identification. It can then be used for species selection prior to strain selection and ecological studies. For bulk biomass, as demonstrated in Fig. 4, low-abundance foliose species within the bulk biomass can be missed. In addition, the presence of species other than foliose *Ulva* within the bulk biomass, for example in *Ulva* blooms containing a mixture of tubular and foliose morphotypes [32], could produce unexpected band patterns. Similarly, the presence of contaminants can only be detected if all species in the bulk biomass can be amplified by the CAPS primers described here. For the highest precision, sensitivity and resolution, the use of next-generation-sequencing (NGS) is required, with the caveat that it requires a significantly higher cost and results turnaround, as well as expertise in the analysis of metagenomics data.

For F2+ hybrid detection, it should be noted, that this assay requires an organellar marker and that it can miss F2+ hybrids if nuclear DNA recombinations during the sexual reproduction of the F1 generation occur outside of the 45S rDNA repeats. In such a case, the individual can be homozygous for the ITS1 locus but heterozygous in other loci in the genome. Hence, the presence of F2+ hybrids is likely to be underestimated using this method. However, sanger sequencing of barcodes would not produce better results, and only NGS [33,34] or microsatellites [35] analysis can conclusively infer the degree of hybridization

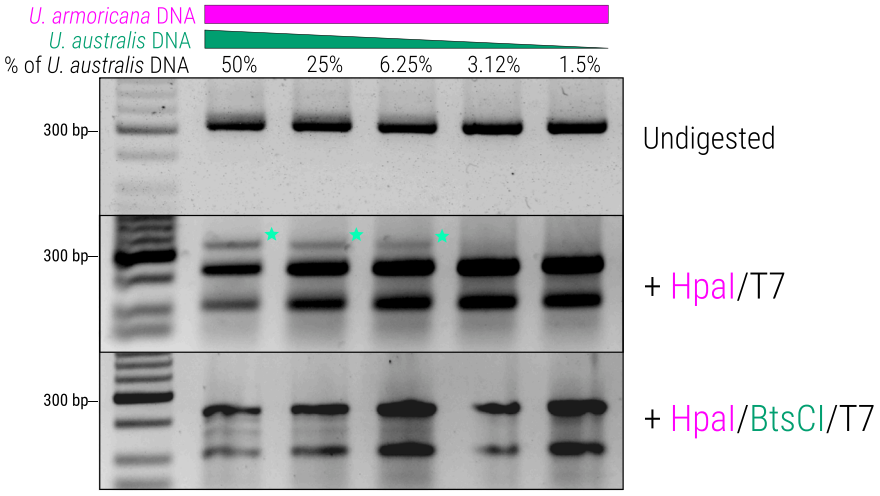

Fig. 4. Sensitivity of the CAPS assay in synthetic bulk samples. DNA of *U. armoricana* and *U. australis* was mixed with decreasing concentration of *U. australis* and detected using digestion of the ITS1 PCR product with HpaI and BtsCI. The presence of an undigested band is indicated by a star.

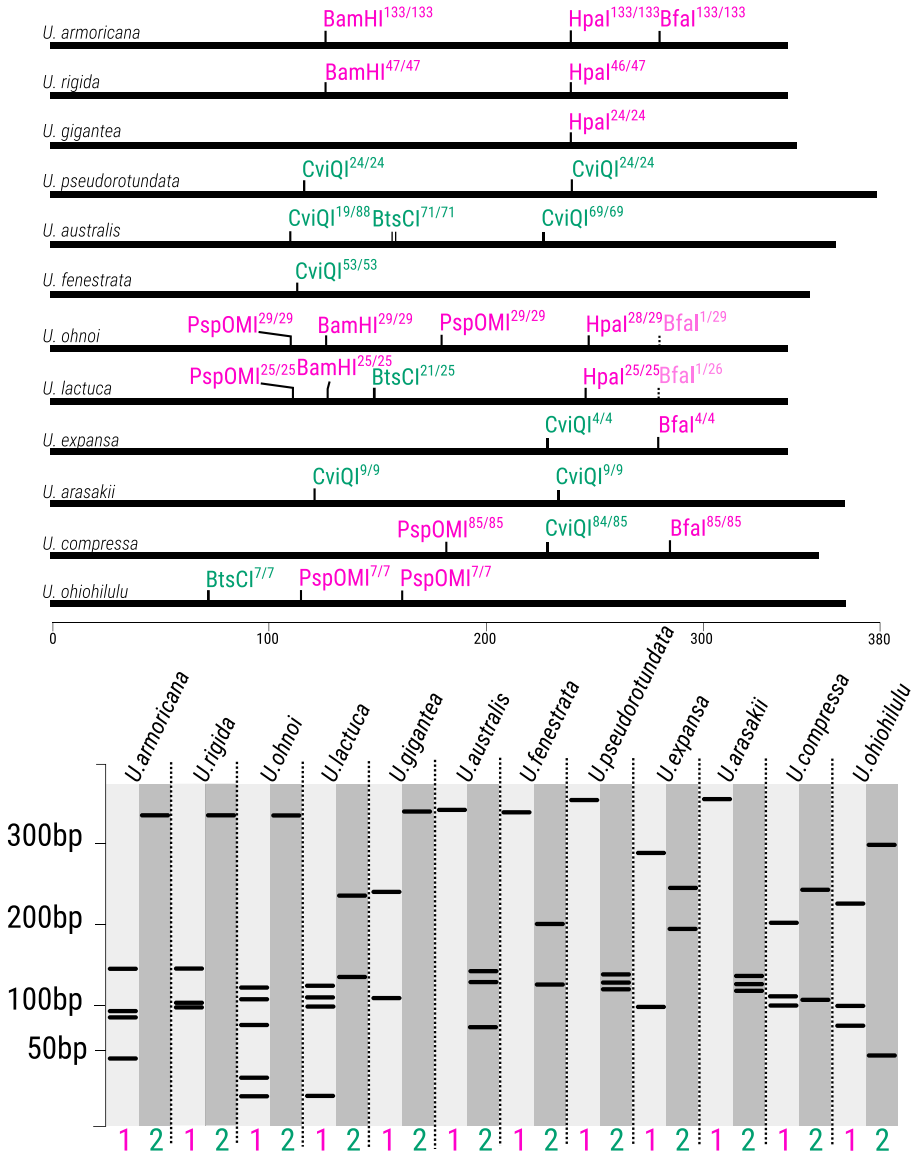

Fig. 5. Prediction of ITS1 digestion pattern of foliose *Ulva* species based on all the ITS1 sequences from the literature. Top: restriction sites are indicated above the lines. Numbers represent the number of individuals of the species harbouring the restriction site. Rare allelic variants are indicated with a pale shading. Bottom: predicted gel results obtained from the digestion of the PCR product with the enzyme mixtures BamHI+BfaI+HpaI+PspOMI (number 1) and CviQI+BtsCI (number 2).

within a population.

Finally, this assay is not aimed at providing higher level resolution than species level given that it uses extremely conserved species-specific SNPs. For the detection of ecotypes/haplotypes/geographical origin of individuals, a combination of several barcodes can be used instead [36,37], or for the highest resolution, next-generation-sequencing [38–40].

4. Conclusions

Our CAPS assay can thus be used to accurately, cheaply and quickly select individuals from a species of scientific or commercial interest during strain selection, or to assess the distribution of foliose *Ulva* species within areas. Importantly, this assay is accessible to any lab with a minimum of molecular biology equipment, as it only requires a PCR machine, and an electrophoresis/gel imaging system. Additionally, this assay is cost-effective as a single reaction containing all enzymes costs €0.50 [US$0.59], compared with ~ €4.00 [US$4.70] per Sanger sequencing reaction using service providers. This assay could be extended to tubular *Ulva* species, and we provide the ITS1 alignment used for validation purposes (containing all *Ulva* ITS1 sequences in the NCBI, excluding those without species information) in Supplementary File 1. The concept of CAPS assay for species identification could also be used on any other taxa of interest for fundamental ecological research and industry traceability purposes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102280.

Funding

This work was funded by the European Union Horizon 2020 programme (project ID 727892, GenialG - GENetic diversity exploitation for Innovative Macro-ALGal biorefinery, http://genialgproject.eu/), SFI Frontiers for the Future (Project Pristine Coasts, grant number 19/FFP/6841) and the European Union Northern Periphery and Arctic Programme (project number 366, SW-GROW - Innovations for Seaweed Producers in the Northern Periphery Area project; http://sw-grow.interreg-npa.eu/).

CRediT authorship contribution statement

Antoine Fort: conceptualization, methodology, writing,

visualisation, formal analysis; **Charlene Linderhof**: formal analysis; **Inés Coca-Tagarro**: formal analysis; **Masami Inaba**: formal analysis; **Marcus McHale**: methodology; **Michael D Guiry**: writing-review & editing; **Kevin Cascella**: project administration; **Philippe Potin**: project administration, funding acquisition; **Ronan Sulpice**: writing, supervision, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank Isabel Azevedo (CIIMAR), Ricardo Bermejo (NUI Galway), Adrie van der Werf and Annelies Beniers (Wageningen University), Paolo Ruggeri (Station Biologique de Roscoff), Helena Abreu (Alga +), Alexandre Berthelot (France Haliotis), Marie-Mathilde Perrineau and Phil Anderson (Scottish Association For Marine Science) for providing some of the strains used in this study.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

References

[1] M.M. Flagella, N. Andreakis, M. Hiraoka, M. Verlaque, M.C. Buia, Identification of cryptic *Ulva* species (Chlorophyta, Ulvales) transported by ballast water, J. Biol. Res. 13 (2010) 47.
[2] H.W. Lee, J.C. Kang, M.S. Kim, Taxonomy of *Ulva* causing blooms from Jeju Island, Korea with new species, *U. pseudo-ohnoi sp. nov.* (Ulvales, Chlorophyta), Algae 34 (2019) 253–266.
[3] I.H. Tan, J. Blomster, G. Hansen, E. Leskinen, C.A. Maggs, D.G. Mann, H. J. Sluiman, M.J. Stanhope, Molecular phylogenetic evidence for a reversible morphogenetic switch controlling the gross morphology of two common genera of green seaweeds *Ulva* and *Enteromorpha*, Mol. Biol. Evol. 16 (1999) 1011–1018.
[4] S. Steinhagen, F. Weinberger, R. Karez, Molecular analysis of *Ulva compressa* (Chlorophyta, Ulvales) reveals its morphological plasticity, distribution and potential invasiveness on German North Sea and Baltic Sea coasts, Eur. J. Phycol. 54 (2019) 102–114.
[5] R. Miladi, A. Manghisi, S.A. Minicante, G. Genovese, S. Abdelkafi, M. Morabito, A DNA barcoding survey of *Ulva* (Chlorophyta), Tunisia and Italy reveals the presence of the overlooked alien U. ohnoi, Cryptogamie, Algologie (2018), https://doi.org/10.7872/crya/v39.iss1.2018.85.
[6] L. Kirkendale, G.W. Saunders, P. Winberg, A molecular survey of *Ulva* (Chlorophyta) in temperate Australia reveals enhanced levels of cosmopolitanism, J. Phycol. 49 (2013) 69–81.
[7] G.W. Saunders, H. Kucera, An evaluation of *rbc*L, *tuf*A, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae, Cryptogam. Algol. 31 (2010) 487–528.
[8] C.J. O'Kelly, A. Kurihara, T.C. Shipley, A.R. Sherwood, Molecular assessment of *Ulva spp*. (Ulvophyceae, Chlorophyta) in the Hawaiian islands, J. Phycol. 46 (2010) 728–735.
[9] S. Steinhagen, R. Karez, F. Weinberger, Cryptic, alien and lost species: molecular diversity of *Ulva sensu lato* along the German coasts of the North and Baltic Seas, Eur. J. Phycol. 54 (2019) 466–483.
[10] A. Fort, M. McHale, K. Cascella, P. Potin, B. Usadel, M.D. Guiry, R. Sulpice, Foliose *Ulva* species show considerable inter-specific genetic diversity, low intra-specific genetic variation, and the rare occurrence of inter-specific hybrids in the wild, J. Phycol. 57 (2020) 219–233.
[11] J.R. Hughey, C.A. Maggs, F. Mineur, C. Jarvis, K.A. Miller, S.H. Shabaka, P. W. Gabrielson, Genetic analysis of the Linnaean *Ulva lactuca* (Ulvales, Chlorophyta) holotype and related type specimens reveals name misapplications, unexpected origins, and new synonymies, J. Phycol. 55 (2019) 503–508.
[12] A. Fort, M. Lebrault, M. Allaire, A.A. Esteves-Ferreira, M. McHale, F. Lopez, J. M. Fariñas-Franco, S. Alseekh, A.R. Fernie, R. Sulpice, Extensive variations in diurnal growth patterns and metabolism among *Ulva spp*. strains, Plant Physiol. 180 (2019) 109–123.
[13] A. Fort, C. Mannion, J.M. Fariñas-Franco, R. Sulpice, Green tides select for fast expanding *Ulva* strains, Sci. Total Environ. 698 (2020), 134337.
[14] A. Fort, M. McHale, K. Cascella, P. Potin, M.-M. Perrineau, P. Kerrison, E. da Costa, R. Calado, M. Domingues, I.C. Azevedo, Exhaustive reanalysis of barcode sequences from public repositories highlights ongoing misidentifications and impacts taxa diversity and distribution: a case study of the Sea Lettuce, Authorea Preprints, (2020).
[15] A. Fort, M.D. Guiry, R. Sulpice, Magnetic beads, a particularly effective novel method for extraction of NGS-ready DNA from macroalgae, Algal Res. 32 (2018) 308–313.
[16] K. Katoh, J. Rozewicki, K.D. Yamada, MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, Brief. Bioinform. 20 (2019) 1160–1166.
[17] S. Capella-Gutiérrez, J.M. Silla-Martínez, T. Gabaldón, trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses, Bioinformatics 25 (2009) 1972–1973.
[18] S. Heesch, J.E.S. Broom, K.F. Neill, T.J. Farr, J.L. Dalen, W.A. Nelson, *Ulva, Umbraulva* and *Gemina*: genetic survey of New Zealand taxa reveals diversity and introduced species, Eur. J. Phycol. 44 (2009) 143–154.
[19] M. Guiry, G. Guiry, AlgaeBase. World-Wide Electronic Publication, National University of Ireland, Galway. 2020, URL: http://www. algaebase. org, (2020).
[20] J.R. Hughey, P.W. Gabrielson, C.A. Maggs, F. Mineur, K.A. Miller, Taxonomic revisions based on genetic analysis of type specimens of Ulva conglobata, laetevirens U., pertusa U., spathulata U., (Ulvales, Chlorophyta), Phyco. Res. (2020), https://doi.org/10.1111/pre.12450.
[21] G. Coat, P. Dion, M.-C. Noailles, B. De Reviers, J.-M. Fontaine, Y. Berger-Perrot, S. Loiseaux-De Goër, *Ulva armoricana* (Ulvales, Chlorophyta) from the coasts of Brittany (France). II Nuclear rDNA ITS sequence analysis, Eur. J. Phycol. 33 (1998) 81–86.
[22] P. Dion, B. De Reviers, G. Coat, *Ulva armoricana* sp. nov.(Ulvales, Chlorophyta) from the coasts of Brittany (France). I. Morphological identification, Eur. J. Phycol. 33 (1998) 73–80.
[23] T. Bråten, Autoradiographic evidence for the rapid disintegration of one chloroplast in the zygote of the green alga *Ulva mutabilis*, J. Cell Sci. 12 (1973) 385–389.
[24] Y. Kagami, Y. Mogi, T. Arai, M. Yamamoto, K. Kuwano, S. Kawano, Sexuality and uniparental inheritance of chloroplast DNA in the isogamous green alga *Ulva compressa* (Ulvophyceae), J. Phycol. 44 (2008) 691–702.
[25] C. Mitsuhashi, H. Teramura, H. Shimada, Construction of genomic marker sets based on the chloroplast genome of a green alga, Ulva pertusa (syn. Ulva australis), leads to simple detection of Ulva species, Genes Genetic Sys. (2020) 19-00054, https://doi.org/10.1266/ggs.19-00054.
[26] A.H. Buschmann, C. Camus, J. Infante, A. Neori, Á. Israel, M.C. Hernández-González, S.V. Pereda, J.L. Gomez-Pinchetti, A. Golberg, N. Tadmor-Shalev, Seaweed production: overview of the global state of exploitation, farming and emerging research activity, Eur. J. Phycol. 52 (2017) 391–406.
[27] J.R. Thompson, L.A. Marcelino, M.F. Polz, Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR', Nucleic Acids Res. 30 (2002) 2083–2088.
[28] J.A. Klappenbach, P.R. Saxman, J.R. Cole, T.M. Schmidt, rrndb: the ribosomal RNA operon copy number database, Nucleic Acids Res. 29 (2001) 181–184.
[29] S.O. Rogers, A.J. Bendich, Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer, Plant Mol. Biol. 9 (1987) 509–520.
[30] O. De Clerck, S.M. Kao, K.A. Bogaert, J. Blomme, F. Foflonker, M. Kwantes, E. Vancaester, L. Vanderstraeten, E. Aydogdu, J. Boesger, G. Califano, B. Charrier, R. Clewes, A. Del Cortona, S. D'Hondt, N. Fernandez-Pozo, C.M. Gachon, M. Hanikenne, L. Lattermann, F. Leliaert, X. Liu, C.A. Maggs, Z.A. Popper, J. A. Raven, M. Van Bel, P.K.I. Wilhelmsson, D. Bhattacharya, J.C. Coates, S. A. Rensing, D. Van Der Straeten, A. Vardi, L. Sterck, K. Vandepoele, Y. Van de Peer, T. Wichard, J.H. Bothwell, Insights into the evolution of multicellularity from the sea lettuce genome, Curr. Biol. 28 (2018) 2921–2933, e2925.
[31] M. Chihara, *Ulva arasakii*, a new species of green algae: its life history and taxonomy, Bull, Nat. Sci. Mus. Tokyo 12 (1969) 849–861.
[32] M. Guidone, C.S. Thornber, Examination of *Ulva* bloom species richness and relative abundance reveals two cryptically co-occurring bloom species in Narragansett Bay, Rhode Island, Harmful Algae 24 (2013) 1–9.
[33] P. Zhao, H.-J. Zhou, D. Potter, Y.-H. Hu, X.-J. Feng, M. Dang, L. Feng, S. Zulfiqar, W.-Z. Liu, G.-F. Zhao, Population genetics, phylogenomics and hybrid speciation of Juglans in China determined from whole chloroplast genomes, transcriptomes, and genotyping-by-sequencing (GBS), Mol. Phylogenet. Evol. 126 (2018) 250–265.
[34] P.A. Hohenlohe, S.J. Amish, J.M. Catchen, F.W. Allendorf, G. Luikart, Next-generation RAD sequencing identifies thousands of SNPs for assessing hybridization between rainbow and westslope cutthroat trout, Mol. Ecol. Resour. 11 (2011) 117–122.
[35] J. Zhang, N. Yotsukura, A. Jueterbock, Z.-M. Hu, J. Assis, C. Nagasato, J. Yao, D. Duan, Detecting no natural hybridization and predicting range overlap in *Saccharina angustata* and *Saccharina japonica*, J. Appl. Phycol. 33 (2021) 693–702.
[36] P.-G. Sauriau, M. Dartois, V. Becquet, F. Aubert, V. Huet, M. Bréret, A. Viricel, E. Pante, Multiple genetic marker analysis challenges the introduction history of Ulva australis (Ulvales, Chlorophyta) on French coasts, Eur. J. Phycol. (2021) 1–13.
[37] T.T. Bringloe, H. Verbruggen, G.W. Saunders, Unique biodiversity in Arctic marine forests is shaped by diverse recolonization pathways and far northern glacial refugia, Proc. Natl. Acad. Sci. 117 (2020) 22590–22596.
[38] M.W. Horton, A.M. Hancock, Y.S. Huang, C. Toomajian, S. Atwell, A. Auton, N. W. Muliyati, A. Platt, F.G. Sperone, B.J. Vilhjálmsson, Genome-wide patterns of

genetic variation in worldwide *Arabidopsis thaliana* accessions from the RegMap panel, Nat. Genet. 44 (2012) 212–216.

[39] K. Xu, X. Yu, X. Tang, F. Kong, Y. Mao, Organellar genome variation and genetic diversity of Chinese *Pyropia yezoensis*, Front. Mar. Sci. 6 (2019).

[40] J. Guzinski, M. Ballenghien, C. Daguin-Thiébaut, L. Lévêque, F. Viard, Population genomics of the introduced and cultivated Pacific kelp *Undaria pinnatifida*: marinas—not farms—drive regional connectivity and establishment in natural rocky reefs, Evol. Appl. 11 (2018) 1582–1597.