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Applications in Plant Sciences

PROTOCOL NOTE

An optimized CTAB method for genomic DNA extraction from green seaweeds (Ulvophyceae)

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Abstract

Premise: Seaweeds are gaining substantial research interest, particularly for genomic applications, where high-quality DNA is a prerequisite. Extracting DNA from these organisms presents challenges due to high levels of biomacromolecules resulting from their diverse cell structures. Existing protocols often lack versatility, leading to inconsistent outcomes across various materials and taxa, which highlights the need for a universal method for use with a variety of green seaweed samples.

Methods and Results: We optimized the conventional cetyltrimethylammonium bromide (CTAB) protocol for green seaweed DNA extraction. Our method, involving an initial sample treatment, lysis buffer adjustment, and enzyme incubation alterations, outperformed the conventional CTAB and commercial kits in terms of DNA yield and purity. Notably, the protocol's effectiveness was demonstrated across various green algal materials and preservation methods, and was tested with downstream applications with satisfactory results.

Conclusions: Our optimized CTAB protocol offers a reliable solution for high-quality genomic DNA extraction from a wide variety of green seaweed samples.

KEYWORDS

absorbance ratios, high-molecular-weight DNA, macrophyte, marine, seaweed

Green seaweeds (mostly ulvophycean algae) are extensively used for fundamental and applied molecular research (Wodniok et al., 2011; Cremen et al., 2018; De Clerck et al., 2018; Umen, 2020). For reliable genomic analyses, high-quality DNA is a prerequisite (Graham et al., 2015; Rhoads and Au, 2015); however, extracting DNA from algae, including green seaweeds, has proven to be a challenging task due to their diverse biochemical cellular structures. These structures include various biomacromolecules, such as polysaccharides, pigments, and phenolics, which pose significant obstacles to the DNA extraction process (Doyle and Doyle, 1990; Domozych et al., 2012; Greco et al., 2014). Consequently, a protocol suitable for isolating DNA from one strain may fail with another, highlighting the need for a versatile protocol that can effectively work with a variety of green algal samples.

Numerous DNA extraction protocols exist, including methods leveraging organic extraction (Sambrook and Russell, 2001), sodium dodecyl sulfate (SDS) (Marmur, 1961), cetyltrimethylammonium bromide (CTAB) (Doyle and Doyle, 1987), salt extraction (Aljanabi and Martinez, 1997), or commercial kits (Snirc et al., 2010; Greco et al., 2014; Hanyuda et al., 2016; Wilson et al., 2016). The suitability of these methods for specific organisms varies, as each has varying degrees of success depending on the target organism. Many factors influence this success rate, including whether the samples are of freshwater or marine origin, their age, and the preservation method used (silica-dried, ethanol, or as herbarium samples). Other method-specific challenges include cost and the need for specialized instruments (Eland et al., 2012; Jagielski et al., 2017; Gumińska et al., 2018). For instance, the UltraClean Soil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, California, USA) offers a protocol for rapid DNA extraction from microalgae; however, this method requires specialized equipment, including a MOBIO vortexer and adapter for the mechanical lysis step (Simonelli et al., 2009; Eland et al., 2012).

Many researchers have focused on CTAB methods for green algal DNA extraction efforts because the protocol is known for its efficacy in handling high-polysaccharide contents, rigid cell walls, proteins, and other inhibitory substances (Doyle and Doyle, 1987; Michiels et al., 2003; Sharma et al., 2008; Tiwari et al., 2012). The reagents used in CTAB-based DNA extractions are also relatively inexpensive, making the method cost-effective for large-scale DNA isolation. Researchers often modify the CTAB protocol by incorporating additional salts, detergents, polymers, reducing agents, and/or adjusting their standard initial sample amounts to optimize DNA extractions for different taxonomic groups (Michiels et al., 2003; Cremen et al., 2016; Schenk et al., 2023). The addition of SDS as an extra detergent has been utilized to break tough cell components and protein bonds, as well as to degrade polysaccharides for easier removal (Ramachandran et al., 2022; Schenk et al., 2023). Additionally, 2-mercaptoethanol, used as a reducing agent, has been effective in reducing other contaminants, although the required concentration varies depending on the target organism (Inglis et al., 2018; Pookhamsak et al., 2019). The lysis step, involving maceration and incubation with a detergent to lyse cells, is highly variable among published CTAB extraction protocols (Schenk et al., 2023), emphasizing that this is a key step in obtaining high-quality DNA.

Many of these modifications have not been tested systematically in either fresh cultures of green seaweeds or those preserved in silica gel or ethanol. Our goal was to optimize a CTAB protocol by incorporating SDS detergent, sodium sulfite (Na_2SO_3) salt, and 2-mercaptoethanol, and altering the initial CTAB concentration and the amount of CTAB buffer. With these modifications, we sought to deliver consistently high-quality DNA extractions from diverse green algal samples with a particular focus on green seaweeds, the Ulvophyceae.

METHODS AND RESULTS

Algae samples

We conducted detailed protocol comparisons for culture strains of four species representative of green seaweed genera: an undescribed *Derbesia* Solier species (strain West 4838), *Halimeda opuntia* (L.) J.V.Lamouroux (voucher HV06932), *Ulvella viridis* (Reinke) R.Nielsen, C.J.O'Kelly & B.Wysor (strain West 4866), and an unidentified *Blastophysa* Reinke strain (Wetherbee BR39). We also tested our optimized protocol on another 20 green algal taxa (Appendix S1). This diverse set encompasses both microscopic algae and macroscopic seaweeds, and includes both fresh cultures and different types of preserved materials, providing a comprehensive evaluation of the optimized extraction protocol.

DNA extractions

We optimized the CTAB method originally described by Doyle and Doyle in 1987, drawing inspiration from the works of Ramachandran et al. (2022), Cremen et al. (2016), Pookhamsak et al. (2019), and Drábková (2014). By systematically testing and optimizing the concentrations of the chemical components of the lysis buffer, along with the introduction of additional reagents, we refined the protocol to achieve satisfactory results in terms of DNA purity and yield. Additionally, we included three additional methods for comparison: conventional CTAB, the commercial Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA), and the Wizard HMW DNA Extraction Kit (Genomic DNA Purification System; Promega), following the manufacturers' instructions.

The full optimized protocol is detailed in Appendix 1, with modifications briefly described here. Initially, 40 mg of each sample was ground into a fine powder using a micropestle and placed in a 2.0-mL tube, which was immersed in liquid nitrogen to avoid thawing. Subsequently, 800 μL of lysis buffer (pre-warmed to 62°C), which consisted of 2% CTAB, 1% polyvinylpyrrolidone, 0.25% SDS, 0.25% sodium sulfite (Na_2SO_3), 100 mM Tris-HCl, 1.4 M NaCl, and 20 mM ethylenediaminetetraacetic acid (EDTA), was added and mixed thoroughly. Following 1 h of incubation at 62°C, 15 μL Prot-K (20 mg/mL) was added and immediately inverted several times. After another 50-min incubation at the same temperature, the mixture was allowed to cool down to 56°C, and 3 μL RNase-A (10 mg/mL) was added and incubated for an additional 30 min.

The samples were then allowed to return to room temperature and washed with an equal volume of chloroform:isoamyl alcohol (24:1). For problematic samples, such as those with consistently low-purity DNA after the aforementioned steps, 0.5–1% 2-mercaptoethanol was added to the extractions. Additionally, the wash step was repeated using phenol:chloroform:isoamyl alcohol (25:24:1) instead of chloroform:isoamyl alcohol (24:1). Otherwise, the DNA precipitation steps were followed by ethanol wash steps, and 50 μL of nuclease-free water was added with a 15-min incubation at room temperature.

Quality assessment

The extracted DNA yield was quantified using a Qubit dsDNA HS assay kit on a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The DNA purity was evaluated using ultraviolet–visible (UV–Vis) spectrophotometry (UV5Nano; Mettler-Toledo, Columbus, Ohio, USA), with absorbance ratios at 260/280 nm (A_{260}/A_{280}) and 260/230 nm (A_{260}/A_{230}). To assess DNA integrity, the samples underwent 2% gel electrophoresis alongside a ≥1 kbp marker ladder. Additionally, we used the TapeStation system (Agilent Technologies, Santa Clara, California, USA) with Genomic DNA ScreenTape to estimate the DNA fragment size distribution. The TapeStation results were produced one year after the DNA extraction following storage at −80°C.

Triplicate extractions were performed for protocol comparison. Statistical analyses, including analyses of variance (ANOVAs) and Tukey's multiple pairwise comparison tests, were conducted using R version 4.4.0 (R Core Team, 2024), with significance levels set at $P < 0.05$ and $P < 0.01$, respectively. Data visualization for the purity metrics was carried out using the ggplot2 package in R (Wickham, 2016), while GraphPad Prism version 10 (GraphPad, Boston, Massachusetts, USA) was used to plot the DNA concentrations.

Results

The performance of our optimized CTAB extraction method was compared with three other protocols using representatives of four green seaweeds. The optimized method yielded the highest average DNA for each strain, with values of 24.1, 25.4, 33.5, and 50.1 ng/μL for the four species (Figure 1). The conventional CTAB protocol yielded slightly lower amounts (22.2, 22.4, 28.4, and 43.8 ng/μL, respectively) than the optimized CTAB method, but the differences were not statistically significant (Appendix S2). By contrast, the commercial kits resulted in significantly lower yields (Figure 1). The optimized CTAB protocol consistently provided satisfactory purity, with an A_{260}/A_{280} of ~1.8 and an A_{260}/A_{230} of ~2.1 (Figure 2). The conventional CTAB method resulted in good ratio results for *Derbesia* alone (~1.8 and ~1.9, respectively), while commercial kit–based extractions exhibited the lowest performance for all samples (Figure 2; Appendices S3 and S4). Of the additional 20 specimens, the calcifying green seaweed (*Halimeda opuntia*) and two herbarium samples (i.e., *Pseudobryopsis* Berthold sp. [voucher MAS135] and *Trichosolen* Montagne sp. [voucher PH591]; Appendix S1) posed a challenge for obtaining pure DNA, which was overcome by adding 0.5–1% 2-mercaptoethanol and repeating the addition of phenol:chloroform:isoamyl alcohol (25:24:1).

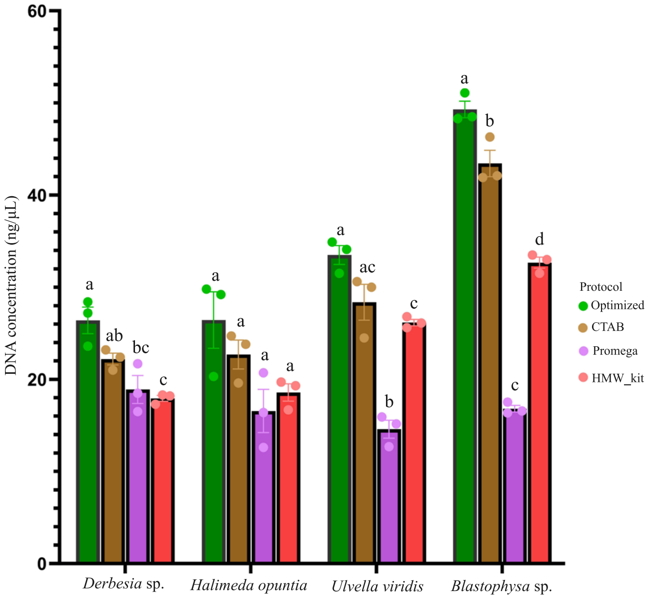

FIGURE 1 Comparison of DNA yields extracted from four green seaweed species using four different extraction protocols: optimized CTAB (Optimized), standard CTAB (CTAB), the Promega Wizard Genomic DNA Purification Kit (Promega), and the Promega Wizard HMW DNA Extraction Kit (HMW_kit). For each species, different letters represent significant differences (at $P < 0.05$ and $P < 0.01$, see Appendix S2), as determined using a one-way ANOVA followed by Tukey's multiple pairwise comparison test. Error bars indicate the standard deviation of the mean concentration.

The integrity of the extracted DNA was satisfactory in the 2% agarose gel, with samples displaying high-molecular-weight DNA, although the *Ulvella viridis* DNA exhibited slight fragmentation (Figure 3). The TapeStation results showed that the overall DNA fragment size was similar for these four species (Appendix S5). *Derbesia* and *Halimeda* exhibited uniformly higher amounts of longer DNA fragments (i.e., ~7 kbp to ≥35 kbp), whereas the *Ulvella* and *Blastophysa* DNA contained a considerable amount of intermediate-sized fragments (i.e., 900 bp to 4 kbp) along with the longer DNA fragments.

The optimized protocol was applied to a further set of 20 green algal samples, encompassing diverse fresh and preserved materials, including ethanol, silica, and herbarium specimens, representing both micro and macro sizes. These algae represent three major groups of green algae (Chlorophyta). Among these, the DNA yield was comparatively higher (~60 ng/μL) from fresh culture samples, except for *Kraftionema* Wetherbee & Verbruggen sp. (17 ng/μL), while the lowest yield was obtained from the herbarium samples (9.2–14.3 ng/μL). Our optimized CTAB protocol consistently delivered satisfactory results in terms of purity (A_{260}/A_{280} of 1.7–1.9 and A_{260}/A_{230} of 2.0–2.2), except for one herbarium sample, *Caulerpa brownii* (C.Agardh) Endl. (A_{260}/A_{280} of 1.6).

CONCLUSIONS

Obtaining high-quality DNA is the primary and most critical step in molecular biology studies, especially when attempting to apply genomic methods to challenging materials such as green algae. This study presents an optimized approach for DNA extraction from a wide range of green algae, particularly focusing on the Ulvophyceae (i.e., green seaweeds), in which we refined the CTAB lysis buffer composition originally published by Doyle and Doyle in 1987. While traditional CTAB methods require sample-specific optimizations, this protocol offers a more versatile solution for both fresh and preserved samples.

To minimize DNA degradation, this optimized protocol adheres to general precautions throughout the extraction process. These precautions include minimizing vortexing, employing gentle pipetting techniques, and avoiding

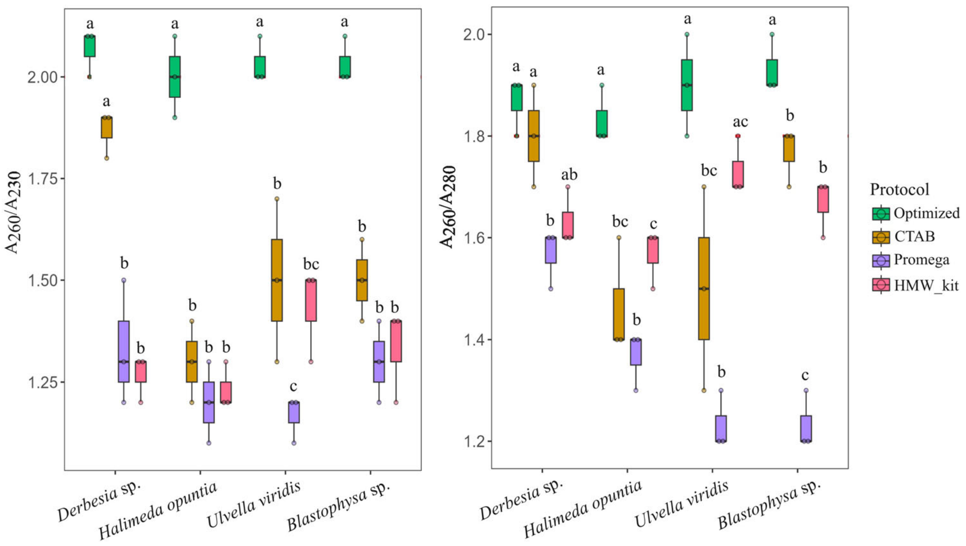

FIGURE 2 Absorbance ratios of the DNA extracted using four different extraction protocols, optimized CTAB (Optimized), standard CTAB (CTAB), the Promega Wizard Genomic DNA Purification Kit (Promega), and the Promega Wizard HMW DNA Extraction Kit (HMW_kit), measured using UV–Vis spectrophotometry (UV5Nano, Mettler-Toledo). (A, B) Absorbance ratios at (A) A_{260}/A_{230} and (B) A_{260}/A_{280}. For each species, different letters represent significant differences (at $P < 0.05$ and $P < 0.01$, see Appendix S2), as determined using a one-way ANOVA followed by Tukey's multiple pairwise comparison test. Error bars indicate the standard deviation of the mean absorbance ratio.

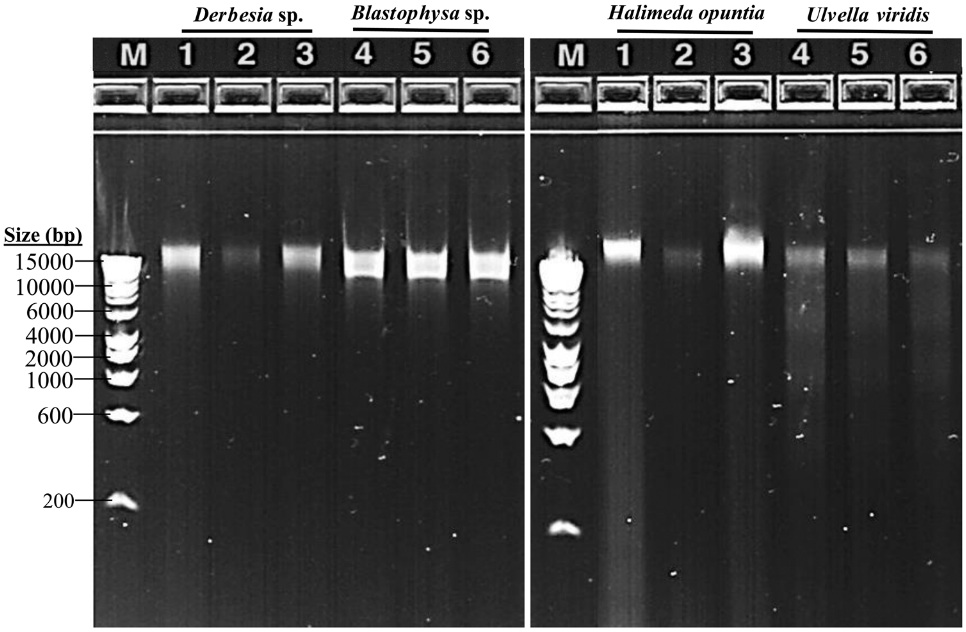

FIGURE 3 Gel electrophoresis of DNA extracted from four studied samples using the optimized CTAB method. Column M represents the ≥1 kbp DNA marker. Three replicates were run for each species, and a total of 3 μL of DNA was loaded into each well.

freeze–thaw cycles during sample preparation. Longer incubation times can enhance enzyme activity and facilitate DNA dissociation from macromolecules in the lysis buffer, thereby increasing DNA yield. However, it is crucial to consider the shelf life and optimal incubation temperatures of the enzymes (Healey et al., 2014; El-Ashram et al., 2016), necessitating careful observation and adjustment to ensure optimal extraction efficiency in accordance with the manufacturer's instructions. Overall, the total incubation times of the conventional CTAB and optimized CTAB protocols are 2 to 2.5 h, whereas commercial kits have much shorter incubation times (typically ranging from 35 min to 1 h), which may exacerbate the differences in yield observed here (Figure 1).

Given the structural diversity of green algae cells, method optimization for one taxon in any protocol often fails for other taxa. The inclusion of 0.25% SDS and 0.25% Na_2SO_3 in the lysis buffer serves to denature proteins, polyphenols, and other macromolecules. The ratio of the initial sample amount to the lysis buffer also contributes to the efficacy of the lysis reaction with the cell components. We therefore believe that our increased volume of initial lysis buffer and the addition of two extra compounds in this protocol enhanced the cellular lysis in a variety of sample types, facilitating the digestion of macromolecules and ensuring DNA purity. For problematic calcifying samples and some herbarium samples, adding 0.5–1% 2-mercaptoethanol and repeating the wash step with phenol:chloroform:isoamyl alcohol (25:24:1) significantly improved the DNA purity, a strategy that has been shown to be effective in high polyphenol contexts (Yee et al., 2018; Heikrujam et al., 2020). This protocol thus ensures satisfactory purity within a range of 1.8–2.1 (at both A_{260}/A_{280} and A_{260}/A_{230}) for calcifying and non-calcifying green seaweeds (where values below 1.8 at A_{260}/A_{280}, and below 2.0 at A_{260}/A_{230} typically indicate carry-over contaminations; Desjardins and Conklin, 2010) (Figure 2). The relatively higher yield of long-fragment DNA from this method (Figure 3, Appendix S5) indicates its potential for use in long-read DNA sequencing platforms.

Numerous protocols exist for DNA extraction from various types of fresh and preserved plant samples. Noteworthy protocols for silica and herbarium-preserved samples include those by Carey et al. (2023), Azeyanty et al. (2020), and Drábková (2014). For ethanol-preserved samples, Johnson et al. (2023) and Bressan et al. (2014) offer well-established methods. However, there was a lack of extraction protocols specifically tailored to green seaweed samples that could effectively handle a variety of sample types. In our study, we applied this optimized protocol to an additional set of 20 green algal samples, encompassing diverse fresh and preserved materials, including ethanol, silica, and herbarium specimens, representing both microalgae and seaweeds. This provided consistently satisfactory results, indicating the versatility of the protocol regardless of sample preservation type. Overall, this optimized CTAB protocol could serve as an inexpensive and universal DNA extraction method for a large group of green seaweeds along with other green microalgae.

AUTHOR CONTRIBUTIONS

R.H. and T.B. devised the optimization plan. R.H. executed the work, conducted the data analysis, and authored the manuscript. M.C. contributed to the extraction process and collaborated on gel documentation with A.S. M.A.K.K. participated in data visualization. H.V. and T.B. provided supervision and made critical edits to the manuscript. H.V. acquired the funding. All authors approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

All data are provided in the Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. List of algae samples used in this study to trial and compare the performance of the optimized CTAB protocol.

Appendix S2. One-way ANOVA with Tukey HSD results for the comparison of DNA yields among four seaweed species across four DNA extraction treatments: (A) optimized CTAB, (B) conventional CTAB, (C) Promega kit, and (D) a Promega high-molecular-weight kit.

Appendix S3. One-way ANOVA with Tukey HSD results for the comparison of the spectral absorbances at 260 and 280 nm of DNA extracted from four seaweed species across four DNA extraction treatments: (A) optimized CTAB, (B) conventional CTAB, (C) Promega kit, and (D) a Promega high-molecular-weight kit.

Appendix S4. One-way ANOVA with Tukey HSD results for the comparison of the spectral absorbances at 260 and 230 nm of DNA extracted from four seaweed species across four DNA extraction treatments: (A) optimized CTAB, (B) conventional CTAB, (C) Promega kit, and (D) a Promega high-molecular-weight kit.

Appendix S5. Size distribution of the DNA extracted from four green seaweed species using the optimized CTAB protocol, as measured using TapeStation one year after the DNA extraction and storage at −80°C.

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Appendix 1. Detailed optimized CTAB extraction protocol and the consumables required.

1. Lysis buffer preparation: 2% CTAB, 1% polyvinylpyrrolidone, 0.25% SDS, 0.25% sodium sulfite (Na_2SO_3), 100 mM Tris-HCl, 1.4 M NaCl, and 20 mM ethylenediaminetetraacetic acid (EDTA). The lysis buffer should be prepared in advance of DNA extraction and can be stored for several months at room temperature. It is crucial to ensure the pH of the lysis buffer is between 7.0 and 8.0.
2. Initial sample amount: ~40 mg.
3. Sample preparation:
    - (i) If any water or ethanol is present in samples, remove it by absorbing with blotting paper.
    - (ii) Transfer the ~40-mg samples into 2-mL Eppendorf tubes and flash-freeze them several times with liquid nitrogen.
    - (iii) For macroalgae, grind samples into a fine powder using a micropestle, avoiding thawing.

      Alternative: For microalgae suspensions, remove as much culture media as possible, add 50 μL lysis buffer, and grind carefully with a micropestle without flash-freezing.
    - (iv) Immediately add 800 μL (750 μL if 50 μL was added for the above optional step) pre-warmed lysis buffer and mix thoroughly.

Comment: To avoid freeze-thawing, we recommend using a 2.0-mL Eppendorf tube to contain the sample, briefly immersing it in liquid nitrogen for a few seconds while keeping the lid open, and gently grinding the sample using a micropestle. Repeat this process multiple times to create a fine powder without allowing the samples to thaw. Immediately after, add pre-heated CTAB before starting the heat incubation.

4. Incubation and enzyme addition:
    - (i) Incubate the prepared samples at 62°C for 1 h, inverting every 7–10 min.
    - (ii) Add 15 μL Prot-K (20 mg/mL) and immediately invert the tubes a few times.
    - (iii) Incubate for 50 min at 62°C, inverting every 7–10 min.
    - (iv) Set the temperature to 56°C and allow 10 min for samples to cool down from 62°C.
    - (v) Add 3 μL RNase-A (10 mg/mL), immediately invert a few times, and incubate for 30 min at 56°C.
    - (vi) Incubate for 10 min at room temperature.
5. DNA separation from other macromolecules:
    - (i) Add an equal volume of chloroform:isoamyl alcohol (24:1) and mix thoroughly.
    - (ii) Centrifuge at 13,000 rpm at room temperature for 10 min.
    - (iii) Carefully transfer the clear upper layer to a new 2.0-mL Eppendorf tube.

TABLE A1 List of consumables required for the extraction of DNA from green seaweeds.

Consumable	Abbreviation	Supplier
Cetyltrimethylammonium bromide	CTAB	MilliporeSigma
Polyvinylpyrrolidone	PVP	MilliporeSigma
Sodium dodecyl sulfate	SDS	MilliporeSigma
Sodium sulfite	Na_2SO_3	MilliporeSigma
Trisaminomethane hydrochloride	Tris-HCl	Thermo Fisher Scientific
Sodium chloride	NaCl	Thermo Fisher Scientific
Ethylenediaminetetraacetic acid	EDTA	Thermo Fisher Scientific
Proteinase K	Prot-K	Thermo Fisher Scientific
Ribonuclease A	RNase-A	Thermo Fisher Scientific
Chloroform:isoamyl alcohol	CIA	MilliporeSigma
2% Mercaptoethanol	2-ME	MilliporeSigma
Phenol:chloroform:isoamyl alcohol	PCIA	MilliporeSigma
80% Isopropanol	IPA	MilliporeSigma
80% Ethanol	EtOH	MilliporeSigma
Nuclease-free water		Thermo Fisher Scientific
Wizard Genomic DNA Purification Kit		Promega
Wizard HMW DNA Extraction Kit		Promega
Qubit dsDNA HS assay kit		Thermo Fisher Scientific

Optional: If working with calcifying or otherwise problematic green algae, add 0.5–1% 2-mercaptoethanol and gently invert a few times, then repeat steps 5(i)–(iii) using phenol:chloroform:isoamyl alcohol (25:24:1) instead of chloroform:isoamyl alcohol (24:1).

Comment: For some herbarium materials and calcified *Halimeda opuntia*, we achieved better results when using phenol:chloroform:isoamyl alcohol.

6. DNA precipitation:
    - (i) Add a two-thirds volume of ice-cold 80% isopropanol and gently mix by inverting a few times, and incubate at −20°C for 15–30 min.
    - (ii) Centrifuge at 13,000 rpm for 10 min at room temperature.
    - (iii) Carefully pipette and discard the supernatant without disturbing the pellet.
7. DNA ethanol wash:

(i) Add 1–1.5 mL 75–80% ethanol and gently invert 2–3 times.

(ii) Centrifuge at 13,000 rpm for 10 min at room temperature and then discard the supernatant.

(iii) Repeat the above two steps with 100% ethanol and centrifuge for 5 min.

(iv) Carefully remove the ethanol and air-dry for 10 min by inverting the tubes on a clean paper towel.

8. DNA elution:

Add 50 μL nuclease-free water and incubate for 15 min at room temperature. When the pellet has dissolved, the sample is ready for assessment.

Optional: If the pellet is not dissolving, incubate at 50°C for 5–10 min and/or double the volume of nuclease-free water, and gently flick the tube using a finger (Table A1).