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PROTOCOL NOTE

Extraction of high-quality, high-molecular-weight DNA depends heavily on cell homogenization methods in green microalgae

Jordan R. Stark[1], Zoe G. Cardon[1], and Elena L. Peredo[1,2]

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[1] Ecosystems Center, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543, USA

[2] Author for correspondence: elperedo@mbl.edu

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PREMISE: New sequencing technologies have facilitated genomic studies in green microalgae; however, extracting high-quality DNA is often a bottleneck for long-read sequencing.

METHODS AND RESULTS: Here, we present a low-cost, highly transferrable method for the extraction of high-molecular-weight (HMW), high-purity DNA from microalgae. We first determined the effect of sample preparation on DNA quality using three homogenization methods: manual grinding using a mini-pestle, automatic grinding using a vortex adapter, and grinding in liquid nitrogen. We demonstrated the versatility of grinding in liquid nitrogen followed by a modified cetyltrimethylammonium bromide (CTAB) extraction across a suite of aquatic- and desert-evolved algal taxa. Finally, we tested the protocol's robustness by doubling the input material to increase yield, producing per sample up to 20 µg of high-purity DNA longer than 21.2 kbp.

CONCLUSIONS: All homogenization methods produced DNA within acceptable parameters for purity, but only liquid nitrogen grinding resulted in HMW DNA. The optimization of cell lysis while minimizing DNA shearing is therefore crucial for the isolation of DNA for long-read genomic sequencing because template DNA length strongly affects read output and length.

KEY WORDS DNA integrity; long-read sequencing; modified CTAB extraction; Scenedesmaceae.

Chlorophyte microalgae are important primary producers present in virtually every photic habitat, including marine, freshwater, hydro-terrestrial, and terrestrial environments (Domozych et al., 2012). Microalgae have developed physiological and morphological adaptations to survive in the low humidity, high light, and fluctuating temperatures characteristic of terrestrial environments, including those of extreme habitats such as deserts, alpine areas, and polar regions. This naturally occurring diversity, together with suitability for year-round culturing, has placed green microalgae at the forefront of applied research (Metting, 1996) in fields including bioremediation (Ji et al., 2013), CO_2 sequestration (Cheah et al., 2015), heavy metal accumulation (Peña-Castro et al., 2004), biofuels (Brennan and Owende, 2010), biohydrogen (Nagarajan et al., 2017), fertilizers (Renuka et al., 2018), and high-value food supplements and cosmetics (Borowitzka, 2013).

Applied research on microalgae has benefited from our increased knowledge of algal genomics, beginning with the publication of the first annotated green algal genomes of *Ostreococcus tauri* C. Courties & M.-J. Chrétiennot-Dinet (Derelle et al., 2006) and *Chlamydomonas reinhardtii* P. A. Dang. (Merchant et al., 2007) just over a decade ago. Since then, more than 114 green algal genomes have become available in the National Center for Biotechnology Information (NCBI) assembly database (https://www.ncbi.nlm.nih.gov/assembly/ query "Chlorophyta" [Organism], consulted February 2020). This rapid growth was possible because of the reduction in costs and the development of third-generation high-throughput sequencing technologies, such as SMRT sequencing (PacBio, Pacific Biosciences, Menlo Park, California, USA) and nanopore (Oxford Nanopore Technologies, Oxford, United Kingdom). These long-read technologies bypass several of the challenges in assembling green algal genomes, including co-occurring bacterial sequences; the presence of nuclear, mitochondrial, and chloroplast DNA; and high GC content (Blaby et al., 2014). Instead of relying on assemblies, long reads span through repetitive regions and low-complexity genomic regions. Long reads, which are able to resolve complete bacterial genomes in a few reads, are also essential for identifying horizontal gene transfer events in eukaryote genomes.

Long-read sequencing technologies require large quantities (1 μg to 15 μg, depending on the platform and desired read length; https://nanoporetech.com/products/kits, https://www.pacb.com/wp-content/uploads/SMRTbell-Library-Preparation-for-High-Fidelity-Long-Read-Sequencing-Customer-Training.pdf) of high-purity, high-molecular-weight (HMW) DNA (Rhoads and Au, 2015). These concentrations of HMW DNA can be particularly challenging to obtain from green microalgae. Microalgal cells are usually small (often <10 μm), have rigid cell walls, and are rich in compounds such as chlorophyll a and b, xanthophylls, beta carotene, starch, and cellulose (Lewis and McCourt, 2004), which deeply influence the DNA extraction process, affecting cell lysis and downstream applications such as PCR amplification (Eland et al., 2012; Greco et al., 2014). The extraction of DNA from terrestrial algae, and especially desert-evolved taxa, is notoriously difficult, likely due to the development of enlarged cell walls during their adaptation to terrestrial environments (Cardon et al., 2008).

Traditionally, methods to improve the quality of extracted genomic DNA have focused on purity and yield, as these parameters have the most impact in the success of downstream applications (hybridization, PCR, activities of restriction enzymes). The purity of samples can be increased by fine-tuning extraction protocols based on the cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987) or by selecting species-appropriate extraction buffers (Tear et al., 2013). Several commercially available kits using proprietary buffers or columns have also been developed to address the difficulty in isolating high-purity DNA from plants including green microalgae (Eland et al., 2012). Yields can be increased by using maxi-prep approaches, by modifying the amount of input material, and by using commercial kits; however, these methods may require specialized equipment not present in every laboratory (such as refrigerated ultracentrifuges) and can become increasingly expensive. Another successful and popular approach for increasing yield is to use strong cell and tissue homogenization methods such as those based on agitation with microbeads (Fawley and Fawley, 2004). Automated homogenization has become a standard step in DNA extraction protocols coupled with second-generation sequencing platforms, characterized by read sizes under 1 kbp (454 sequencing, Roche, Basel, Switzerland; SOLiD, Illumina, San Diego, California, USA); however, bead-based homogenization methods mechanically damage DNA. The resulting low-molecular-weight DNA is not suitable for third-generation sequencing platforms (Gumińska et al., 2018) unless post-extraction size selection steps are completed (e.g., dedicated magnetic bead kits or gel-based systems such as BluePippin [Sage Science, Beverly, Massachusetts, USA]).

Here, we present a low-cost, highly scalable DNA extraction protocol specifically designed for extracting high-quality, HMW DNA suitable for use with next-generation long-read sequencing technologies. Our approach, which we successfully demonstrate in a variety of green microalgae, optimizes cell lysis to increase yields while maintaining DNA integrity. First, we compared three methods for homogenizing and disrupting microalgal cells prior to DNA extraction, with the aim of maximizing the yield of HMW DNA without compromising purity. Then, we validated the suitability of our extraction method for application to a broad range of taxa. We tested the method in a suite of green microalgae within the Scenedesmaceae (Chlorophyta), which have specialized physiologies resulting from adaptation to the drastically different habitats of freshwater environments and desert soils. Finally, we verified the scalability of the method by evaluating the effect of increasing the initial material input on quality parameters.

METHODS AND RESULTS

Microalgal strains

- *Enallax costatus* (Schmidle) Pascher, 1943 (isolate CCAP276-31 from the Culture Collection of Algae and Protozoa)
- *Tetradesmus obliquus* (Turpin) M. J. Wynne, 2016 (isolate Utex 72 from the University of Texas Culture Collection)
- *Acutodesmus deserticola* (L. A. Lewis & Flechtner ex E. Hegewald, C. Bock & Krienitz) E. Hegewald, C. Bock & Krienitz, 2013 (isolate BCP-SNI-2 from L. Lewis, University of Connecticut)
- *Flechtneria rotunda* Sciuto & L. A. Lewis, 2015 (isolate BCP-SEV3-VF49 from L. Lewis, University of Connecticut)

Culturing techniques

Two aquatic (*E. costatus* and *T. obliquus*) and two terrestrial (*A. deserticola* and *F. rotunda*) microalgal species were cultured in 150 mL of growth medium composed of a 1 : 1 mix of Bold's Basal Medium with micronutrients (Bold, 1949) and Woods Hole Medium (Stein et al., 1973). All algal cultures were non-axenic monoisolates. All culturing procedures were carried out under sterile conditions. The cultures were grown in 250-mL Erlenmeyer flasks at 25°C in a Conviron PGW36DE growth chamber (Conviron, Winnipeg, Canada) under a 12-h/12-h light/dark photoperiod and 40 μE light from metal halide and sodium lamps. The cultures were constantly bubbled with ambient air. Fresh medium was added every week by allowing the cells to settle and replacing half of the supernatant (~75 mL) with fresh medium to sustain high rates of cellular division (Fig. 1A). The algal cultures were grown for six weeks before the DNA extractions.

Cell collection and culture preconditioning

For each algal species and flask, we harvested the cells from the 150-mL culture. We adjusted cultures to a density of ~10^7 cells mL^{-1} (determined using a Biotek Synergy HT plate reader; BioTek Instruments, Winooski, Vermont, USA). Algal cells were allowed to settle and the clear supernatant was poured off. The concentrated algal culture was transferred into a 15-mL Falcon tube, where the cells were further concentrated by gravity into a final volume of approximately 2–3 mL. The remaining supernatant was removed, and 500 μL of each highly concentrated culture were transferred into Eppendorf tubes for preconditioning prior to the DNA extraction. The samples were centrifuged for 1 min at 5000 rpm, resulting in the formation of an algal pellet ranging in size (estimated as volume) from 50–100 μL.

A white layer of debris was observed between the algal pellet and the supernatant. The composition of this layer was determined under a microscope to be bacteria and empty cell walls (Fig. 1A), which accumulate during cellular division. These algal species within the Scenedesmaceae divide asexually through multiple fission (Cardon et al., 2018). During this process, a mother cell undergoes multiple rounds of nuclear division followed by cellular division. Once division is completed, the daughter cells are released, leaving the empty cell wall of the mother cell behind (Fig. 1A). To precondition

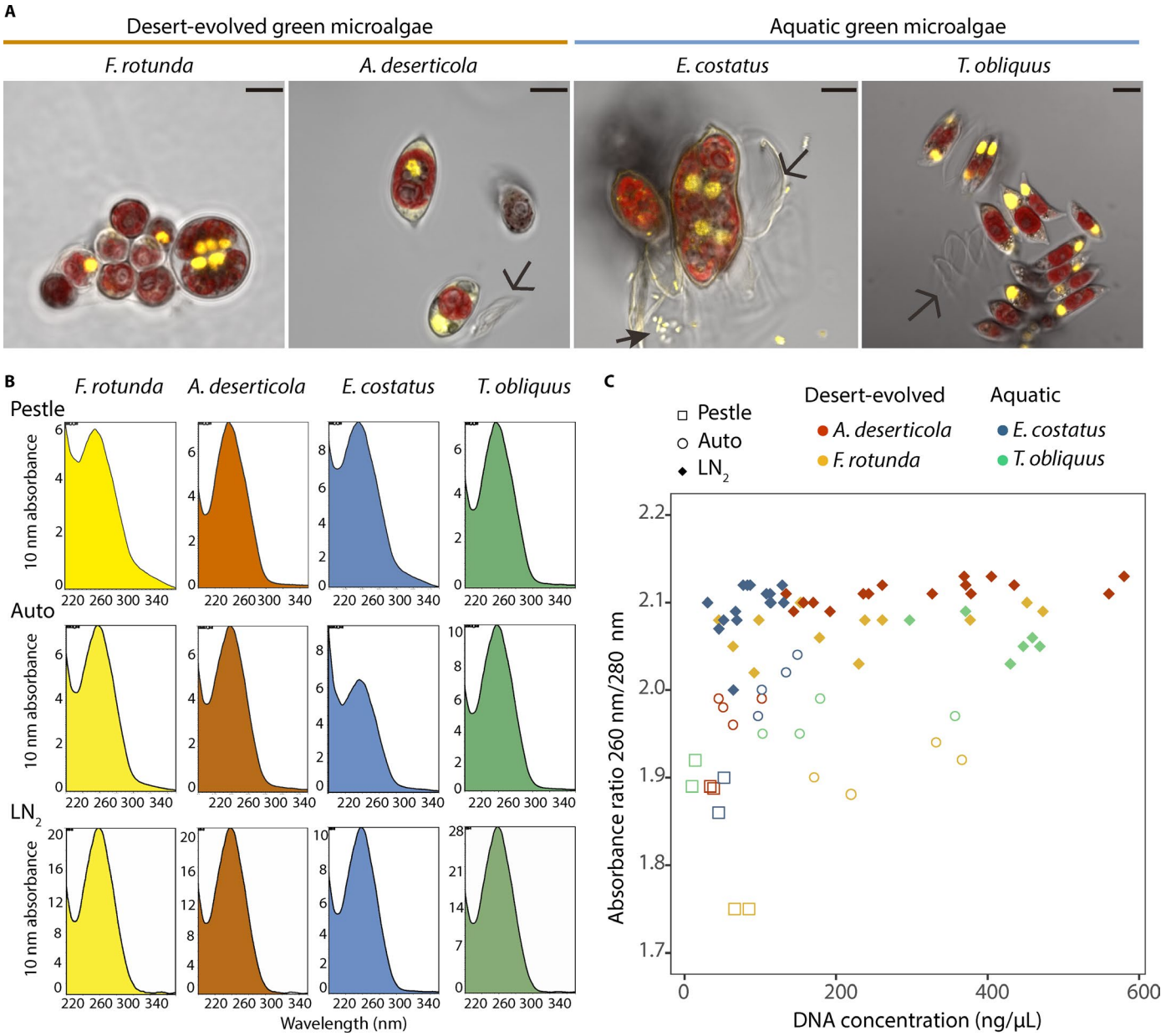

FIGURE 1. Green microalgal species used in this study and the effect of homogenization methods on the purity of their extracted DNA. (A) Laser scanning confocal microscope images of the four microalgal species within the Scenedesmaceae used in this study. Images were captured with a 100× objective in channel mode. The residual light was integrated to generate an optical image. The nuclei were visualized using the dsDNA stain SYBR safe (yellow, 450/50 band pass filter) and the chloroplasts' chlorophyll fluorescence (red, 595/50 band pass filter) (see Cardon et al., 2018 for details). Scale bars = 5 µm. Debris, cell walls (open arrows), and bacteria (closed arrows) are indicated. Multinucleated cells are dividing cells. (B) Spectral patterns representing absorbance of a given sample at different wave lengths (one representative NanoDrop graph per species per treatment). (C) Scatterplots representing DNA quality measured as 260 nm/280 nm absorbance ratios vs. the DNA concentration. Grinding with a mini-pestle (squares) or using an automatic vortex adapter (circles) produced some high-purity, high-concentration samples, but overall the absorbance ratios were lower and more variable. Samples ground in liquid nitrogen (LN_2; filled diamonds) had uniformly high absorbance ratios across species, even when the pellet sizes were big enough to produce >550 ng/µL DNA.

the cultures prior to DNA extraction, the supernatant and this debris layer were removed with a micropipette without disturbing the pelleted cells. The algal pellets were resuspended in 1 mL of fresh sterile medium by gently inverting the tubes. This preconditioning process was repeated two additional times to remove additional debris with varying centrifugation speeds (2500 rpm and again at 5000 rpm; see Appendix 1 for step-by-step protocol).

Cell homogenization

For each algal species, we tested the effects of three commonly used homogenization methods on the quality, molecular weight, and quantity of the extracted DNA. Success in the homogenization of cells was initially estimated by visual inspection of the treated samples under the microscope and by the presence of algal pigments

(e.g., chlorophylls) in the extraction buffer. The three homogenization methods tested were:

Manual grinding with mini-pestles (two samples per species)—One scoop (~75 µL volume) of autoclaved commercial silica sand (40–100 mesh, ACROS Organics; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 100 µL of CTAB extraction buffer (CEB-500-02; OPS Diagnostics, Lebanon, New Jersey, USA) were added to each algal pellet in an Eppendorf tube. Each sample was manually ground with a mini-pestle for approximately 1 min. Resuspending the pellets in small volumes is required during manual grinding because the cells will float, and therefore resuspending in large volumes would decrease the effectiveness of the process. After grinding, 500 µL of CTAB supplemented with 2.5% β-mercaptoethanol (BP176-100; Thermo Fisher Scientific) was added to the cells, for a final concentration of 2% β-mercaptoethanol.

Automatic grinding using a vortex adapter (four samples per species)—One scoop of autoclaved commercial silica sand and 600 µL of CTAB extraction buffer supplemented with 2.5% β-mercaptoethanol were added to each algal pellet in an Eppendorf tube. All samples were placed in a vortex adapter (MoBio 13000-V1-24; QIAGEN, Hilden, Germany) and vortexed at maximum speed for 5 min. To increase cell lysis, the samples were incubated in a heat block at 55–60°C for 20 min, followed by an additional 5 min of vortexing to ensure that enough cells were disrupted.

Grinding in liquid nitrogen (10 samples per species)—After preconditioning, all the algal pellets from a single flask (see preconditioning step) were transferred into a pre-chilled mortar using 100–1000-µL wide-bore pipette tips (tip ends were cut with a sterile blade). If needed, a small volume of the algal medium was added to the pellets to facilitate the transfer (<100 µL). Samples were flash-frozen in liquid nitrogen (LN_2), and each pellet was continuously ground using a mini-pestle until the LN_2 had evaporated but the sample had not thawed. The grinding process was repeated a total of six times, after which all algal material had the consistency of fine powder. The number of repetitions was initially determined by observing the integrity of the cells under a microscope, and it might vary for other algal taxa. A wide-bore pipette tip was used to transfer the homogenized algal material into a sterile Eppendorf tube and centrifuged briefly to collect the sample. No supernatant was removed at this time. The final volume of material in each Eppendorf tube was 50–100 µL. Finally, the samples were frozen in LN_2 and thawed at room temperature five times to lyse any remaining cells. The pellets were gently resuspended in 600 µL of CTAB extraction buffer supplemented with 2.5% β-mercaptoethanol. See Appendix 1 for a detailed outline of this method.

DNA extraction and testing

For the DNA extraction of all samples, regardless of homogenization method, a modified CTAB extraction method (Doyle and Doyle, 1987) was used; see Appendix 1 for details. Throughout the extraction protocol, we recommend taking general precautions to prevent additional DNA fragmentation such as minimizing vortexing, gently pipetting (using wide-bore tips if possible), and avoiding freeze-thaw cycles on extracted DNA.

The homogenized algal cells were incubated in extraction buffer (CTAB supplemented with 2.5% β-mercaptoethanol) at 55–60°C in a thermal block for 1 h. The tubes were allowed to cool and reach room temperature; 700 µL of 25 : 24 : 1 phenol : chloroform : isoamyl alcohol (IB05174; IBI Scientific, Dubuque, Iowa, USA) was then added to each tube and briefly vortexed to mix. The samples were centrifuged at 14,000 rpm for 10 min, and the aqueous upper phase was transferred to a new Eppendorf tube; 4 µL of RNase A (10 mg/mL) (Zymo Research, Irvine, California, USA) was then added to each sample. The samples were incubated at 37°C for 30 min in a thermal block. The samples were again allowed to cool to room temperature before a second wash with 700 µL of phenol : chloroform : isoamyl alcohol and centrifuged. If the samples appeared visibly dirty, this cleaning step was repeated a third time. The remaining upper aqueous phase (~350–450 µL) was transferred to a new Eppendorf tube, and the DNA was precipitated with ~0.1 volumes of 3 M sodium acetate and ~0.7 volumes of cold isopropanol (Sigma-Aldrich, St. Louis, Missouri, USA). The samples were mixed by gently inverting the tubes. Immediately after mixing, the DNA was easily observable in most samples as clear, gelatinous blobs at the bottom of the tube. The samples were stored at −20°C overnight.

Following precipitation, the DNA was pelleted by centrifuging for 5 min at 14,000 rpm. The supernatant was removed, and the DNA pellets were washed twice with 700 µL of 70% ethanol (Thermo Fisher Scientific). After the final centrifugation step, the supernatants were removed, and pellets were air-dried by inverting the tubes on a clean paper towel. The DNA pellets were then resuspended in 45 µL of TE buffer (Thermo Fisher Scientific).

In an additional experiment focused on increasing yield, DNA was extracted from *E. costatus* and *A. deserticola* cultures using the LN_2 homogenization method with double the amount of starting material ("high input"; pellets of ~200 µL). For these high-input samples, the cells used had been previously frozen at −80°C in culturing medium immediately after collection. All other steps were performed without change.

Yield, purity, and integrity of extracted DNA

Yield—The DNA concentration of each sample was determined using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific) in a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Grinding with LN_2 produced the highest average DNA yields of the three methods in *T. obliquus* (412 ng/µL) and *A. deserticola* (228 ng/µL) (Table 1). In *F. rotunda* and *E. costatus*, the automatic grinding produced the most DNA (272 and 121 ng/µL, respectively), followed by grinding with LN_2 (222 and 77 ng/µL, respectively) (Table 1). Grinding samples with mini-pestles resulted in the lowest DNA concentrations in all species (12–76 ng/µL) (Table 1). The high-input samples (with approximately double the starting algal material) produced higher yields; this was especially substantial in *A. deserticola*, with a two-fold change in yield per Eppendorf tube (Table 1).

Purity—DNA absorbance ratios (260 nm/280 nm and 260 nm/230 nm) were determined for all samples using a NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific) (Fig. 1B). As a general rule, values under 1.8 of the 260 nm/280 nm ratio indicate carry-over contamination from protein, carbohydrates, polyphenols, or phenol that could strongly affect downstream

TABLE 1. Quality parameters measured for DNA extracted from cells homogenized using different methods.

		Concentration (Qubit)		Purity (NanoDrop)		Integrity (electrophoresis)
Species (habitat)	Method (*n*)	Mean (ng/μL ± SE)	Total (μg)[a]	260 nm/280 nm (mean ± SE)	260 nm/230 nm (mean ± SE)	Distribution of DNA fragments
Enallax costatus (aquatic)	Pestle (2)	49 ± 4	2.2	1.9 ± 0.02	1.6 ± 0.11	Broad peak
	Auto (4)	121 ± 13	5.4	2.0 ± 0.01	1.2 ± 0.03	Broad peak
	LN_2 (10)	77 ± 11	3.4	2.1 ± 0.01	2.2 ± 0.03	Tight peak
	High input (5)	103 ± 9	4.6	2.1 ± <0.01	1.9 ± 0.02	Tight peak
Tetradesmus obliquus (aquatic)	Pestle (2)	12 ± 2	0.5	1.9 ± 0.02	2.1 ± 0.05	Broad peak
	Auto (4)	198 ± 55	8.9	2.0 ± 0.01	2.1 ± 0.04	Broad peak
	LN_2 (6)	412 ± 27	18.5	2.1 ± 0.01	2.4 ± 0.01	Tight peak
Acutodesmus deserticola (desert)	Pestle (2)	35 ± 1	1.5	1.9 ± <0.01	2.1 ± 0.03	Broad peak
	Auto (4)	66 ± 13	2.9	2.0 ± 0.01	2.0 ± 0.04	Broad peak
	LN_2 (10)	228 ± 27	10.2	2.1 ± <0.01	2.3 ± 0.01	Tight peak
	High input (6)	448 ± 41	20.1	2.1 ± <0.01	2.3 ± 0.01	Tight peak
Flechtneria rotunda (desert)	Pestle (2)	76 ± 10	3.4	1.8 ± <0.01	1.3 ± 0.03	Broad peak
	Auto (4)	272 ± 46	12.2	1.9 ± 0.01	1.8 ± 0.05	Broad peak
	LN_2 (12)	222 ± 42	9.9	2.1 ± 0.01	2.2 ± 0.03	Tight peak

Note: n = number of samples.
[a]For each sample, the total volume was 45 μL.

applications. Values of ~1.8 are considered pure DNA, whereas values over 1.8 can be associated with changes in nucleotide ratios, especially increases in adenine content (chloroplast genomes, for example, are adenine rich [Smith, 2012]), or the presence of RNA. If present, RNA can be observed easily in agarose gels and removed by means of additional RNase A treatment of samples followed by ethanol precipitation. We did not observe presence of RNA when our samples were checked on agarose gels (see Fig. 2A, C for positive and negative images of representative gels), so no additional RNase A treatments were performed. For the 260 nm/230 nm ratio, values under 2.0 indicate presence of contaminants. For all the treatments, the 260 nm/280 nm ratios ranged between 1.8 and 2.1. The absorbance ratios of those DNAs extracted by grinding with mini-pestles were consistently lower (1.8–1.9) than those extracted using automatic grinding or LN_2 (1.9–2.1) (Table 1, Fig. 1C). For all species, the 260 nm/230 nm ratio was over 2 in samples homogenized with LN_2, as is recommended for long-read sequencing. In *E. costatus* and *F. rotunda*, lower 260 nm/230 nm ratios were observed for samples extracted using other lysis methods.

The absorbance ratios were compared across treatments using an ANOVA performed in R (R Development Core Team, 2011) (Table 1). When pooling all species, different homogenization methods produced significantly different ratios of absorbance at 260 nm/230 nm ($F_{2,70}$ = 26.88, P < 0.001) and at 260 nm/280 nm ($F_{2,70}$ = 165.8, P < 0.001). LN_2 grinding produced the highest 260 nm/230 nm ratios for all species (1.9–2.4; see Table 1), including in those samples in which the initial amount of algal material had been doubled (Table 1).

Integrity—The molecular weight of the extracted DNA was determined by running 2 μL of each sample in 0.8% agarose gel, 1× TAE buffer (Thermo Fisher Scientific) for 4 h at 60 V. The DNA was stained with 1× SYBR safe (Invitrogen, Waltham, Massachusetts, USA). The molecular weight of the genomic DNA was estimated by comparison with a HMW DNA ladder (Lambda DNA/*Eco*RI+*Hind*III; Thermo Fisher Scientific). Digital images of the gels were generated in an Amersham 600 RGB imager (GE Healthcare, Chicago, Illinois, USA) using automatic collection parameters. The images of the gels were enhanced (contrast, homogenization, and background removal) in ImageJ version 1.52a (Rasband, 2018) prior to the digital analysis. The 1D gel electrophoresis image analysis software GelAnalyzer2010a (www.gelanalyzer.com) was used to create profiles of the distribution of DNA fragments (Fig. 2A, B) using the Lambda DNA/*Eco*RI+*Hind*III DNA ladder as a size reference.

For all species, HMW DNA was exclusively observed in the DNA samples extracted from cells homogenized using the LN_2 grinding method. The DNA extracted using this treatment was observed as a tight, clear band over the 21.2-kbp marker band, whereas DNA extracted from cells homogenized using the other treatments displayed substantial smearing and lacked a clear HMW DNA band, consistent with high DNA fragmentation (Fig. 2).

CONCLUSIONS

Current genome sequencing protocols rely on a combined approach of short (Illumina) and long (PacBio, Nanopore) reads. The efficiency of long-read sequencing is directly impacted by the integrity of the DNA used. Our results indicate that for all algal species tested here, a modified CTAB protocol is sufficient for extracting DNA within reasonable quality parameters. However, DNA integrity is strongly affected by the cellular homogenization method used early in the extraction protocols (i.e., during the lysis step). DNA extracted using automatic or mini-pestle grinding is suitable for PCR or short-read sequencing but not for long-read sequencing technologies. Grinding cells in LN_2 was the only homogenization method that consistently resulted in HMW DNA.

Many potential modifications to DNA extraction methods and kits can produce acceptable results for different sequencing methods; however, they can be much more expensive and less customizable than CTAB-based extraction protocols. Our results demonstrate that, for a diverse suite of microalgae taxa that includes aquatic species and desert-derived species with recalcitrant characteristics for DNA extraction, sample preparation and cell lysis methods were key to producing high-quality DNA. Across the four species, uniformly good results were obtained from the CTAB extraction after grinding the cells in LN_2, even though the initial samples varied in cell size, cell wall thickness, and buoyancy. We also

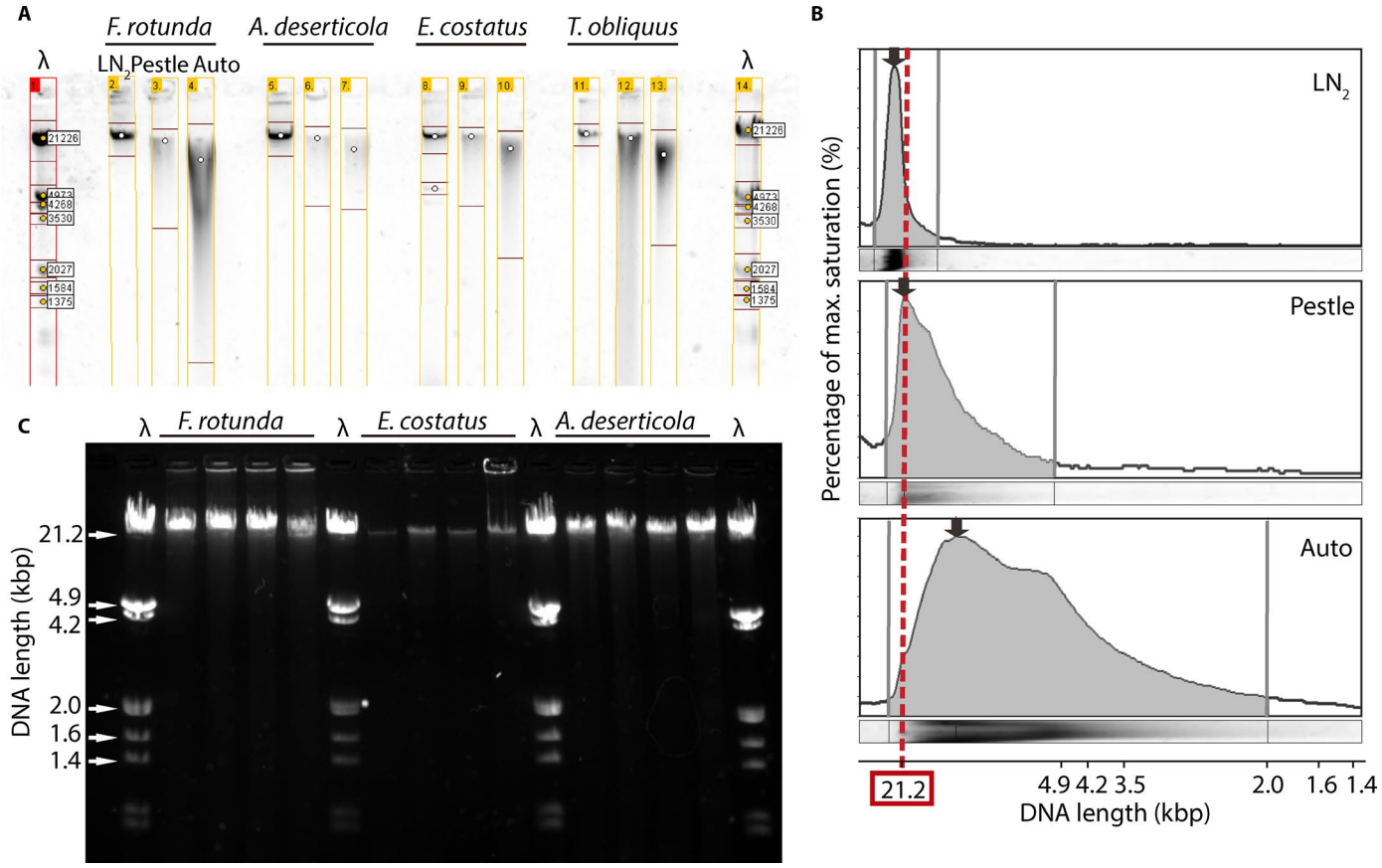

FIGURE 2. Effect of homogenization method on DNA integrity. (A) Gel image analysis of the DNA produced from all four species following their cellular homogenization using liquid nitrogen (LN_2) grinding, mini-pestle grinding, and automatic vortex grinding. The image analysis was performed in GelAnalyzer2010a. (B) Saturation plots (% of max) for the three gel electrophoresis lanes. The results presented correspond to DNA extracted from *Flechtneria rotunda* and are representative of those observed for the other taxa. Extraction following LN_2 homogenization produces a sharp peak of DNA of over 21.2 kbp in size, whereas manual grinding with a mini-pestle and automatic vortexing methods resulted in major peaks of DNA with a considerably lower molecular weight. The broad distribution of DNA fragments is consistent with degraded DNA. (C) Examples of multiple DNA extractions using LN_2 grinding followed by modified CTAB extraction in several green microalgae. Each lane was loaded with 2 µL of extracted DNA. In all cases, LN_2 grinding produced a clear, high-molecular-weight band. The *Tetradesmus obliquus* samples had similarly sharp bands (as shown in A) but were not included in this gel for ease of loading. For all gels, λ indicates the ladder lane (Lambda DNA/*Eco*RI+*Hind*III).

demonstrated that this method can produce up to 20 µg of DNA in a single Eppendorf tube without sacrificing purity or quality, using fresh or frozen material.

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AUTHOR CONTRIBUTIONS

E.L.P. and Z.G.C. designed the experiments; E.L.P. and J.R.S. acquired and analyzed the data; E.L.P. interpreted the data; J.R.S. and E.L.P. wrote the manuscript; and J.R.S., Z.G.C., and E.L.P. approved the submitted version.

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APPENDIX 1. DNA extraction protocol: Sample homogenization using liquid nitrogen and modified CTAB method.

1. Preconditioning of cultures
    a. Concentrate algal cultures in the minimum volume possible and aliquot (500 µL) into Eppendorf tubes for preconditioning
    b. Centrifuge algal cells in growth medium at 5000 rpm for 1 min
    c. Carefully remove the supernatant and white interface layer (cell walls and bacteria) with a micropipette
    d. Add 1 mL of fresh sterile growth medium and resuspend cells
    e. Centrifuge at 2500 rpm for 30 s
    f. Repeat steps c–d
    g. Centrifuge at 5000 rpm for 30 s
    h. Repeat step c, removing as much supernatant as possible
2. Cell grinding with liquid nitrogen (LN_2)
    a. Autoclave mortar and pestle to sterilize
    b. Pre-chill mortar and pestle with LN_2
    c. Resuspend algal culture in as little medium as possible (here <100 µL) and transfer to pre-chilled mortar using a wide-bore tip (cut tip end with sharp, sterile blade)
    d. Grind with pestle until LN_2 has evaporated but cells have not thawed
    e. Add a small amount of additional LN_2
    f. Repeat steps d and e five more times (cells should look damaged under microscope)
    g. Transfer algal material to Eppendorf tubes and centrifuge briefly to collect sample in bottom
    h. Freeze and thaw (at room temperature) centrifuge tubes five times in LN_2
3. DNA extraction
    a. Prepare extraction buffer with CTAB and 2.5% β-mercaptoethanol. Add enough CTAB- β-mercaptoethanol to each Eppendorf tube to bring the total volume to 600 µL
    b. Incubate samples at 55–60°C
    c. Add 700 µL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and vortex for 3–5 s to mix
    d. Centrifuge for 10 min at 14,000 rpm
    e. Transfer aqueous phase to new sterile Eppendorf and add 4 µL of RNase A
    f. Incubate for 30 min at 37°C
    g. Repeat steps b–d and transfer aqueous phase to new sterile Eppendorf; repeat twice if samples still appear visibly dirty
    h. Add 0.1 volumes of cold 3 M sodium acetate and 0.7–0.9 volumes of cold isopropanol. Mix by inversion.
    i. Precipitate overnight at −20°C
    j. Centrifuge for 5 min at 14,000 rpm
    k. Remove supernatant
    l. Add 700 µL of cold 70% ethanol to wash DNA pellet
    m. Centrifuge for 1 min at 14,000 rpm
    n. Repeat steps k–m
    o. Remove supernatant and invert Eppendorf tubes on a clean paper towel to air-dry pellets
    p. Resuspend in 45 µL of TE buffer in refrigerator at least overnight