

NOTE

GENOMIC DNA ISOLATION FROM GREEN AND BROWN ALGAE (*CAULERPALES* AND *FUCALES*) FOR MICROSATELLITE LIBRARY CONSTRUCTION¹

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A method for isolating high-quality DNA is presented for the green algae *Caulerpa* sp. (*C. racemosa*, *C. prolifera*, and *C. taxifolia*) and the brown alga *Sargassum muticum*. These are introduced, and invasive species in Europe, except for the native *C. prolifera*. Previous methods of extraction, using cetyl trimethyl ammonium bromide or various commercial kits, were used to isolate genomic DNA but either no DNA or DNA of very low quality was obtained. Genomic libraries were attempted with *Caulerpa* sp. on three occasions and either the restriction enzyme, the Taq polymerase, or the T4 ligase was inhibited, probably by the large amount of polysaccharides in these algae. The method presented here consists of the rapid isolation of stable nuclei, followed by DNA extraction. Yields of 6–10 µg genomic DNA from 1 g fresh blades were obtained. After genomic DNA was isolated from fresh material, the quality was checked by agarose gel. Quantification of DNA concentration was performed using UV spectrophotometric measurement of the A_{260}/A_{280} ratio. The DNA was suitable for PCR, cloning, and hybridization. The DNA isolated using this method allowed successful construction of microsatellite libraries for *Caulerpa* species and *S. muticum*. The technique is inexpensive and appropriate for the isolation of multiple samples of DNA from a small amount of fresh material.

Key index words: algae; *Caulerpa*; CTAB; DNA extraction; microsatellite; nuclei isolation; population genetics; *Sargassum*; seaweed

The isolation of high-molecular-weight DNA that is suitable for the construction of microsatellite libraries or in any genomic library and in general for digestion with restriction endonucleases, cloning, hybridization, PCR amplification can represent a serious problem in many organisms. The isolation of DNA from plants and algae is quite difficult (Doyle and Doyle, 1987). Furthermore, a procedure that works with one a plant or an algal group will often fail with others, probably because of the diversity of cell wall, storage, and secondary compounds (Doyle and Doyle, 1990), complicating the preparation of nucleic acids from specific groups of these organisms. Many of these compounds inhibit down-stream enzymatic reactions (Huang et al. 2000).

The extraction of DNA from seaweed cells that are heavily embedded in sulfated polysaccharides (cell walls and intercellular matrix) is complicated and time consuming. Most of the published methods for DNA extraction from green algae (Meusnier et al. 2004), red algae (Hong et al. 1997, Waittier et al. 2000), and brown algae (Phillips et al. 2001) require grinding tissues in liquid nitrogen. Viscous soluble polysaccharides are released by grinding algal material in liquid nitrogen (Brasch et al. 1981) that are difficult to separate from the DNA, therefore cetyl trimethyl ammonium bromide (CTAB) treatments (e.g. Fawley and Fawley 2004), cesium chloride (CsCl)-gradient ultracentrifugation (La Claire et al. 1997, Phillips

¹Received 23 September 2005. Accepted 14 February 2006.

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et al. 2001), or lithium chloride (LiCl) (Hong et al. 1992, 1997) methods have been applied during DNA extraction.

Caulerpales are green algae that have been shown to act as invasive species in the Mediterranean, where two exotic *Caulerpa* species, *Caulerpa taxifolia* (M. Vahl) C. Agardh and *Caulerpa racemosa* (Forsskål) J. Agardh (migrant from the Red Sea, de Villèle and Verlaque, 1995), have spread into areas formerly occupied by seagrasses. *Caulerpa prolifera* (Forsskål) J.V. Lamouroux is a worldwide *Caulerpa* species and it is the only indigenous *Caulerpa* sp. in European coasts. *Sargassum muticum* (Yendo) Fensholt is an invasive brown alga that has been introduced in Europe from Japan and presently ranges from the Mediterranean to Norway (Verlaque 2001). More than 100 nuclear rDNA ITS sequences from *C. taxifolia* and other *Caulerpa* species, as well as for *Sargassum* species, are available from GenBank. These sequences have proven valuable in clarifying phylogenies and identifying some biogeographical divisions (Famà et al. 2002, Stiger et al. 2003, Meusnier et al. 2004). Previous attempts to study the population genetic diversity of *Caulerpa* species have failed because the molecular markers used were not polymorphic enough to assess the level and spatial pattern of genetic variability efficiently among populations. In order to design new markers, the construction of libraries with high-quality genomic DNA for these invasive species was being undertaken.

The coenocytic nature (multinucleate) of the green algae *Caulerpa* makes extraction of their DNA more difficult. Isolation using standard methods (CTAB, LiCl, commercial kits, etc) either failed, or the DNA was of poor quality or very degraded. With the above methods, inhibition of the restriction, polymerase, or ligase enzymes occurred when attempting to construct libraries. Moreover, the small size of the nuclei and the absence of internal cell walls (*Caulerpa* sp. is composed of giant cells) imply the use of an average of 4–5 g of material for obtaining 0.2–0.3 µg of DNA. Finally, some of the available methods (e.g. CsCl long-term gradient centrifugation) are costly.

We devised a new approach to extract DNA in recalcitrant algae consisting in rapid isolation of nuclei, followed by DNA extraction. Isolation of stable nuclei was based on the method of Triboush et al. (1998) to isolate chloroplast and mitochondria in sunflower seedlings. The following protocol is inexpensive, reproducible, and can be used for extraction of genomic DNA for microsatellite library construction.

Algal material. The species used in this study were collected at Mallorca (Spain), Algarve (Portugal), and Gulf of Naples (Italy) (Table 1). The method was first developed for three species of *Caulerpa* (*C. taxifolia*, *C. racemosa*, and *C. prolifera*) and then it was tested in *S. muticum*. Seaweed samples were collected in the field and were held at 4° C for a few days before DNA isolation.

Isolation of nuclei. 4 to 10 g of fresh material was homogenized in the mortar with 50 mL STE buffer (400 mM sucrose, 50 mM Tris pH 7.8, 20 mM EDTA-Na₂, 0.2% bovine serum albumin, 0.2% β-mercaptoethanol, and the last two components were added just before the start of the experiment). The homogenate was filtered through a 50–55 µm nylon mesh, closing the mesh to form a bag and squeezing by hand to extract the liquid. The extract was centrifuged at 1000 rpm for 20–30 min. The supernatant was discarded and the nuclei pellet was collected.

DNA isolation procedure. Approximately 500 µL CTAB buffer (2% CTAB, 2% polyvinylpyrrolidone, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris HCl pH 8.0) was added to the large green nuclei pellets. The sample was heated in a water bath or thermoblock at 65° C for approximately 1 h. One volume of chloroform–isoamyl alcohol (24:1) was added to the sample and mixed by inversion for 10 min and then centrifuged for 30 min at 13,200 rpm. The aqueous phase was collected into a clean microcentrifuge tube and the rest was discarded. Two volumes of absolute ethanol were added with 0.1 volume (approximately 50 µL) of sodium acetate 3 M pH 5.2 and mixed gently. The sample was left for at least 20 min at –20° C and afterwards centrifuged for 30 min at 13,200 rpm. The supernatant was discarded and the pellet was washed in 70% ethanol and dried at room temperature. The pellet was dissolved in 10–50 µL of pure water or TE (1 ×) buffer (1 mM Tris HCl pH 8.0, 0.1 mM EDTA pH 8.0). After genomic DNA was isolated from fresh material, the quality and the size of DNA were checked by agarose gel against a known molecular marker (Fig. 1). Quantification of DNA concentration was performed by spectrophotometric measurement of UV absorbance (Table 2).

Construction of the genomic libraries. DNA was digested with the restriction enzyme *AfaI*, and ligated to adaptors Rsa21 (5'-TCTTGCTTACGCGTGGACTA-3) and Rsa25 (5'-TAGTCCACGCGTAAGCAAGAG-CACA-3') with T4 Ligase (Promega, Madison, WI,

TABLE 1. Seaweed species used in this study.

Species	Location	Date	Material
<i>Caulerpa prolifera</i>	Mallorca, Spain	15 May 2004	Fresh thalli
<i>C. taxifolia</i>	Mallorca, Spain	15 May 2004	Fresh thalli
<i>C. racemosa</i>	Naples, Italy	15 September 2005	Fresh thalli
<i>Sargassum muticum</i>	Algarve, Portugal	20 August 2004	Fresh thalli

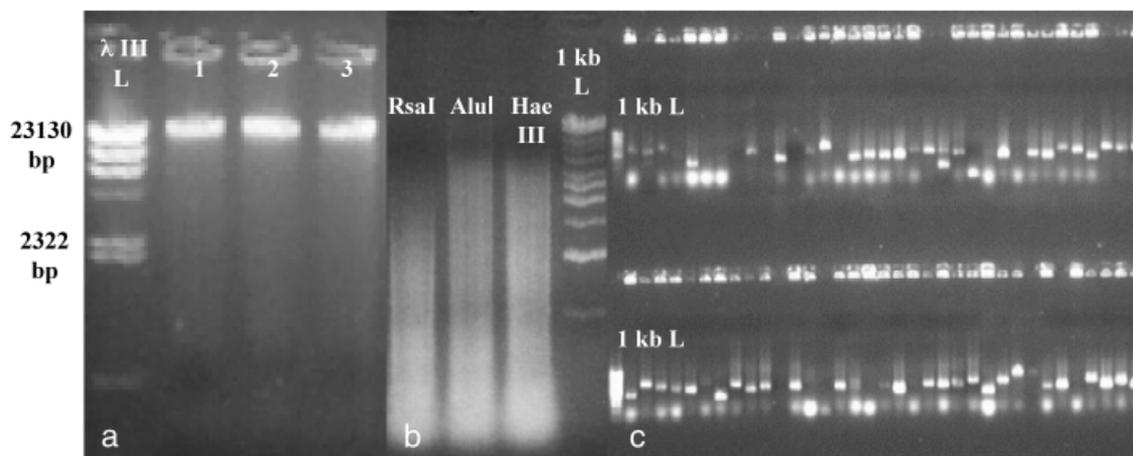


FIG. 1. Electrophoresis analysis (0.8% agarose gel in $10 \times$ TAE buffer (40 mM Tris acetate, 2 mM EDTA)) of (a) three random samples of *Caulerpa prolifera* genomic DNA extracted with the procedure developed here. Samples were collected in (1) Mallorca, (2) Menorca, and (3) Cabrera, Balearic Island, Spain. (b) *C. racemosa* DNA digested with three endonucleases (*RsaI*, *AluI*, and *HaeIII*) according to the manufacturer's instructions, (c) PCR amplification screening of 96 clones of *C. prolifera* with possible dinucleotide microsatellites. The PCR reactions were performed in 20 μ L reaction volume containing buffer $10 \times$, dNTPs (2 mM), $MgCl_2$ (50 mM), universal primers SP6 (5'-CATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACTATAGGG-3') (10 mM), 0.3 U of Taq polymerase, and approximately 5–10 ng of template DNA. The reaction conditions were as follows: 94° C for 3 min, followed by 30 cycles (94° C for 45 s, 50° C for 45 s, and 72° C for 45 s) and then a 3 min final extension at 72° C. 100 L: 100 bp DNA ladder (Fermentas, Ontario, Canada), λ III L: DNA digested with *HindIII* Ladder.

USA). The DNA fragments were then purified with the high-purification kit for PCR products (Amersham Biosciences, Piscataway, NJ, USA) and were successfully amplified by PCR using both adaptors as primers. An enrichment method using the MagneSphere[®] magnetic separation kit (Promega) was then performed to select fragments containing a microsatellite motif among all the DNA fragments contained in the library following the procedure of Waldbieser (1995). This procedure was performed for one motif, (CT)₁₅, with the corresponding 5'-biotinylated and 3'-ddC probe for *C. prolifera*, *C. taxifolia*, and *S. muticum* libraries. For *C. racemosa*, three motifs were used: (CA)₁₅, (GA)₁₅, and (TA)₁₅ (all with the corresponding 5'-biotinylated and 3'-ddC probe). Selected fragments were ligated into pGemT-easy vector (Promega) and then transformed into competent *Escherichia coli* cells (DH5- α) following the manufacturer's protocol. Bacteria colonies were incubated on Petri dishes in Luria-Bertani (LB) agar with ampicillin (100 μ g/ μ L) at 37° C overnight. Recombinant colonies were picked and grown in LB medium with ampicillin (100 μ g/ μ L), iso-

propyl β -D-thiogalactopyranoside (IPTG) (0.1 M), and 5-bromo-4-chloro-5-indolyl- β -D-galactopyranoside (X-Gal) (50 mg/mL) at 37° C for at least 4 h in 96-well plates. Next, 30% of pure glycerol was added and plates were stored at -80° C as stocks.

Screening of the libraries. Denatured and diluted bacteria were used as templates for PCR with plasmid primers SP6 (5'-CATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACTATAGGG-3'). The PCR reactions were performed in a 20 μ L volume containing buffer ($10 \times$), dNTPs (2 mM), $MgCl_2$ (50 mM), universal primers SP6 and T7 (10 mM), 0.3 U of Taq polymerase, and approximately 5–10 ng of template DNA. The reaction conditions were as follows: 94° C for 3 min, followed by 30 cycles (94° C for 45 s, 50° C for 45 s, and 72° C for 45 s), and then a 3 min final extension at 72° C (Fig. 1). The PCR products were then dot-blotted on a nylon membrane and DNA was hybridized (Rapid Hyb, Amersham Biosciences, Buckinghamshire, UK) with a γ -³²P-labeled microsatellite probe. Positive clones were selected and grown in LB agar with ampicillin

TABLE 2. DNA quantification in random samples of DNA when applying our new method for *Caulerpa taxifolia*, *C. racemosa*, and *Sargassum muticum*.

	A_{260}	A_{260}/A_{280}	Purity	Concentration (μ g/ μ L)	Amount of DNA extracted per gram of fresh weight (μ g/g FW)
<i>C. taxifolia</i>	0.221	1.684	84%	0.206	10.3
<i>C. taxifolia</i>	0.204	1.636	81%	0.175	8.75
<i>C. racemosa</i>	0.161	1.75	87%	0.161	6.8
<i>S. muticum</i>	0.226	1.657	82%	0.199	9.95

(100 µg/µL) overnight and then sent for miniprep preparation and sequencing (Macrogen, Seoul, Korea).

The DNA extracted with this method was of high molecular weight with no sign of degradation (Fig. 1). The yield was in the range of 6–10 µg DNA from 1 g of fresh material (Table 2). The procedure did not require gradient centrifugation (sucrose, percol), or ultracentrifugation (CsCl). There were two critical factors in this protocol.

The first one was the recovery of the nuclei pellets, which depends on the speed and length of the centrifugation step, as well as the separation of the nuclei from cell debris and endosymbiotic bacteria associated with these algae. The second critical factor was the use of sodium acetate added to the DNA before precipitation. In this last step, sodium acetate was used instead of NaCl to avoid residual salt problems, which could inhibit DNA ligase (Sambrook et al. 1989).

The DNA was perfectly suitable for digestion with restriction enzymes (such as *Rsa*I, *Alu*I, *Hae*III), PCR, cloning, hybridization, and other molecular techniques used in the construction of the microsatellite libraries (Figs. 1, a and b). In all cases, the efficiency of the transformation was very high, with more than 80% of the bacteria containing inserts. The four libraries were constructed successfully and microsatellites were found in each library following the methods described previously. An average of 6–10 µg of genomic DNA was used per library.

Before radioactive hybridization, PCR amplification with plasmid primers (SP6 and T7) of cloned inserts yielded PCR products of 150–900 bp (Fig. 1c). Hybridization experiments indicated a varying proportion of positive clones within the four libraries. For *C. prolifera*, a total of 960 clones were screened, resulting in 214 positive clones. In *C. taxifolia*, of 768 screened clones, only 23 gave a positive signal and for *C. racemosa*, 120 positive clones were found. In contrast, in *S. muticum*, 672 clones were screened and 382 were positive. After sequencing, the number of microsatellites found in the four species was different, and *Sargassum* had the highest number. In total, 92 microsatellites have been found (30 perfect dinucleotide repeats, 30 compound dinucleotide repeats, nine trinucleotide repeats, two tetranucleotide, and one pentanucleotide). The length of the microsatellites varied from 6 to 24 repeats.

There are several published studies on the phylogeny and the biogeography of *Caulerpa* sp. (Verlaque et al. 2003, Meusnier et al. 2004). All these previous studies used DNA extraction protocols (mostly using the CTAB method) that produced DNA of good enough quality for PCR amplification; however, we found that the quality of DNA was not good enough for genomic library construction. In general, there are several valuable methods in the literature for seaweed DNA extractions, but for some algal taxa these methods yield DNA that is not useful for PCR amplification or restriction enzyme digestion. For example, Hong et al. (1992) developed a simple method using LiCl for

TABLE 3. Repeat units found in clones from *Caulerpa prolifera* libraries.

(GA) ₂₁ , (TC) ₁₈ , (TC) ₈ , (TC) ₉ , (AG) ₂₄ , (GA) ₆ , (GA) ₆ , A ₁₂ (TA) ₅ , (GA) ₆ (AA(GA) ₂)AAG(AA(GA) ₂)
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the rapid extraction of seaweed nucleic acids suitable for PCR analysis. However, the LiCl protocol did not work in the same way in all the tested species. Hong et al. (1997) found that DNA extracted from most seaweed species by the LiCl method were of sufficient quality to be used as a template for PCR amplification, with the exception of DNAs from a few species, which yielded large quantities of DNA, but did not show any PCR product, probably due to the presence of inhibitors of the DNA polymerase. Jin et al. (1997) tested 70 species of brown, red, and green algae for PCR inhibitors. Species such as *Colpomenia bullosa* (Saunders) Yamada, *Sargassum thunbergii* (Mertens ex Roth) Kuntze, *Symphyocladia latiuscula* (Harvey) Yamada, and *Ulva* sp. showed very high inhibitory activity in PCR reactions. This inhibitory activity by cytosolic inhibitors in PCR reactions in DNA extracts of seaweeds has been associated with antiviral and antitumor effects (Kim et al. 1997, Cann et al. 2000, Eitsuka and Nakagawa, 2004).

Before the new method was developed, we tried to construct a microsatellite library in the three species of *Caulerpa*, three times without success, using all the DNA extraction protocols mentioned above. With the method developed here, DNA from these species and *S. muticum* is now suitable for cloning, sequencing, hybridization probe technology, and, consequently, for genomic library construction.

Table 3 shows the dinucleotide repetitions found among positive clones sequenced from the microsatellite-enriched genomic library in *C. prolifera*. This information will be useful to design new molecular markers for population genetic studies of all these species.

In conclusion, the protocol presented is highly recommended for seaweed DNA extractions. The procedure is a combination of two modified protocols developed previously by Triboush et al. (1998) for nuclei isolation, and by Doyle and Doyle (1990) for DNA isolation in land plants. The procedure is rapid, requires few solutions, and is effective in isolating genomic DNA from *Caulerpa* species and *S. muticum*, where other methods failed. As the main advantage, this procedure provides genomic DNA of high quality with no degradation and with high yields from a small amount of material. The DNA isolated by this method has been successfully used in PCR, cloning, hybridization, and in other techniques used in the construction of genomic libraries.

This work has been supported by the project CAULEXPAN (REN2002-00701) to N. M., as well as by the EU Network of Excellence MARINE GENOMICS EUROPE (MGE, EU-FP6 contract no. GOCE – CT. 2004-505403). E.V.-A. was supported

by a postdoctoral fellowship of the Spanish Ministry of Education (EX. 2003-512). We thank A. Engelen for the collection of *S. muticum* in Portugal, and Liam Cronin for his comments on the manuscript.

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