METHODS AND TECHNIQUES

# A rapid and cost-efficient DMSO-based method for isolating DNA from cultured lichen photobionts

**Eva M. del Campo,[1] Alicia del Hoyo,[1] Leonardo M. Casano,[1] Fernando Martínez-Alberola[2] & Eva Barreno[2]**

1 *Department of Plant Biology, University of Alcalá, 28871-Alcalá de Henares (Madrid), Spain*
2 *ICBIBE, Department of Botany, University of Valencia, Faculty of Biology, C/ Dr. Moliner 50, 46100-Burjassot (Valencia), Spain*
Author for correspondence: *Eva M. del Campo, eva.campo@uah.es*

**Abstract** We have developed a simple and fast procedure for the purification of PCR-quality DNA from cultured lichen photobionts. This new one-step method uses the solvent dimethyl sulfoxide (DMSO) combined with heat treatment to chemically breakdown algal and plant tissues. The DMSO-DNA extracts may be directly precipitated and purified by standard techniques in a total time of approximately 30 min. Compared to other DNA extraction protocols, the DMSO-based method suppresses the need for liquid nitrogen, any extraction buffer, grinding, phenol, and long incubation or centrifugation steps, thereby considerably reducing the possibility of contamination. In addition, minimal amounts of starting material ($5 \times 10^6$–$20 \times 10^6$ cells from liquid or agarized cultures) produce sufficient DNA for 200 PCR reactions approximately, making this protocol a practical option for colony screening. This method works well in a wide range of cultured lichen photobionts and reduces the amount of labor-intensive steps and time consumed by other multi-step procedures, allowing for efficient processing of an increased number of samples.

**Keywords** algae; cyanobacteria; DMSO; DNA isolation; lichens; photobiont; plants

## ■ INTRODUCTION

Biodiversity studies and phylogenetic investigations have been facilitated by methods for identifying species based on short standardized DNA sequences, known as "DNA barcodes". DNA barcoding will accelerate the pace of species discovery by allowing taxonomists to rapidly sort specimens, and by highlighting divergent taxa that may represent new species (Hebert & Gregory, 2005). The aid of DNA barcoding in species identification is more remarkable in certain groups of organisms whose identification can be very difficult. The algae as a group are a good example, due to their relatively simple morphology and anatomy, limited phenotypic plasticity, and alternation of heteromorphic generations. Among them, green algae belonging to Chlorophyta are morphologically simple single cells, packets of cells, or weak filaments, which represent a series of taxa spanning the classes Prasinophyceae, Chlorophyceae, Trebouxiophyceae, and Ulvophyceae. To date, it is impossible to assess which morphological characters used for separation of species and genera are phylogenetically significant. Only recently, molecular methods based on DNA analysis have started to reveal the real phylogenetic diversity of green and non-green algae (Friedl, 1995; Huss & al., 2002; Krienitz & al., 2003, 2004; López-Bautista & al., 2006; Yoon & al., 2006; Khan & al., 2007).

Microscopic algae present special problems for DNA isolation, mainly due to the mechanical strength of their cell walls and their small size. An additional problem, which usually causes difficulty for phylogenetic analyses in many organisms, is the scarcity of starting material. For this reason, an increasing number of new micromethods for isolating DNA from diverse biological sources have been developed. Most of them include several hazardous and expensive chemical products and time-consuming steps. DNA extraction techniques commonly used for microalgae and plants include mechanical disruption or by freezing in liquid nitrogen and grinding to powder in a mortar (Doyle & Doyle, 1990; Friedl, 1995; Fawley & Fawley, 2004; Reynolds & Williams, 2004). Dimethyl sulfoxide (DMSO) is a widely used solvent for the extraction of chlorophylls (Chls) from leaves of higher plants and algae (Nikolopoulos & al., 2008). It has been demonstrated that treatment with pectinase or DMSO leads to a loosening of the most internal layers of structure in green algae (König & Peveling, 1984). Herein, we propose a new method for isolating DNA from cultured microscopic algae based on DMSO extraction efficiency combined with a heat treatment, which is also applicable to several other plant species. This DNA extraction procedure requires only 30 minutes, and a minimal amount of starting material ($5 \times 10^6$–$20 \times 10^6$ cells from liquid or agarized cultures of algae or approximately 15 mg of plant tissue).

## ■ MATERIALS AND METHODS

**DNA isolation procedure. —** The new DNA isolation method is suitable for algae grown in both liquid cultures and agarized media, as well as for several plants. For liquid cultures, 100–400 µl ($5 \times 10^6$–$20 \times 10^6$ cells) were centrifuged in a 1.5 ml microfuge tube at 14,000 g for 1 min; the supernatant was discarded and pellets were resuspended by briefly pipetting with an equal volume (vol) of DMSO. For algae grown on agarized media and plant samples, 15 mg of fresh weight (corresponding

to approximately $14 \times 10^6$ cells in the case of algae) were incubated with 400 µl DMSO. In other cases, for evaluating individual algal clones grown on agarized media, colonies were picked with sterile micropipette tips and resuspended in 100 µl DMSO by pipetting. Incubation with DMSO was performed at 65°C for 10 min and was followed by centrifugation at 10,000 g for 5 min. This procedure was sufficient to extract DNA suitable for PCR in all cases. The DMSO-extracted DNA was directly precipitated by two alternative methods which resulted in equally sufficient samples: (1) incubation with 1 vol of 4M ammonium acetate ($NH_4OAc$) and 2 vol of isopropanol for 10 min at room temperature; (2) incubation with 1 vol of 3M sodium acetate pH 5.2 and 2 vol of 100% ethanol for 30 min at –80°C. After centrifugation at 14,000 g for 10 min, at room temperature in (1) or at 4°C in (2), the resulting pellets were washed once or twice with 70% ethanol. After air-drying, the pellets were resuspended in 15–50 µl of TE (pH 8.0) or ultrapure water and were stored at 4°C until use in PCR.

**Tested organisms. —** The selected algal and plant species used in this protocol are listed in the Appendix. All tested algae except for *Trebouxia* sp. were obtained from the following culture collections: "Sammlung von Algenkulturen" at the University of Göttingen (Germany) and the Culture Collection of Algae at the University of Texas at Austin. *Trebouxia* sp. was cultured axenically after isolation in our laboratory from the lichen *R. farinacea* (del Campo & al., 2009). All organisms were cultured either in liquid or on agarized media except *Nostoc insulare* SAG 54.79, which was cultured only in liquid medium. All of them were grown in Bold 3N medium (Bold & Parker, 1962) either with glucose (20 g/l) and casein (10 g/l), or without them in a chamber at 22°C (except *Trebouxia* species which were grown at 17°C) under 14 h/10 h light/dark cycle (lighting conditions: 25 $\mu mol \times m^{-2} \times s^{-1}$). Fresh tissues were used for DNA extraction from several plants from Spain, including *Porella platyphylla* (liverworts) from Cercedilla (Madrid), *Equisetum palustre* (ferns) from the Henares river valley (Guadalajara), and various flowering plants from local nurseries.

**PCR amplification of nrITS and chloroplast-encoded psbA and LSU rDNA genes. —** To evaluate the DNA obtained with the new extraction method for suitability in downstream application(s), isolated DNAs were employed as templates for PCR amplifying several nuclear and chloroplast-encoded genes. The primer pair ITS-1T/ITS-4T, which amplified the internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene and the internal transcribed spacer 2 (ITS2) of the nuclear-encoded ribosomal RNA, was obtained from Kroken & Taylor (2000). The primer pairs psbAchl1/psbAchl2 and 23S-AL1/23S-AL2 were designed in our laboratory for amplifying two chloroplast-encoded genes: the *psbA* encoding for the D1 protein (psbAchl1: 5′-TGG TTA TAC AAC GGT GGT CC-3′ and psbAchl2: 5′-GGA AGT TGT GAG CGT TAC GC-3′) and the LSU rDNA (23S-AL1: 5′-GGG TAG AGC ACT GTT TCG G-3′ and 23S-AL2 5′-CCT TCT CCC GAA GTT ACG G-3′). Amplification reactions were preformed in a total reaction volume of 25 µl using Illustra Hot Start Mix RTG (GE Healthcare, New Jersey, U.S.A.). This system provides reagents for PCR reactions stable at room temperature. The only user-supplied reagents that need to be added are template DNA and specific primers, allowing for improved reproducibility while minimizing the potential for contaminations. Negative controls, without DNA template, were included in every round of PCR amplification to ensure against false-positive results caused by contaminants in the reagents. To control against apparatus-bias, PCR amplifications were performed in two different DNA Thermal Cyclers (BioCycler TC-S, Boeco™, Hamburg, Germany; and SensoQuest Labcycler, Progen Scientific Ltd, South Yorkshire, U.K.). Cycling conditions were as follows: 94°C for 2 min; 40 cycles of 94°C for 30 s, 50°C for 60 s and 72°C for 1 min; followed by a final extension at 72°C for 7 min. Amplification products (8 µl)

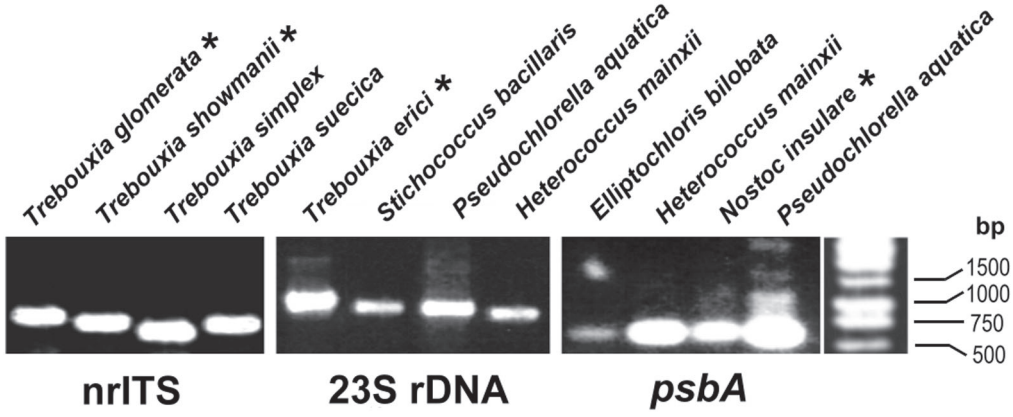


**Fig. 1.** Agarose gel (1.5% w/v) electrophoresis of PCR amplification products using DNA extracted from several algae and some plants (see Table 1) and specific primers for both nuclear and chloroplast genes. Molecular weight markers (1 kb DNA Ladder, Promega Co, Madison, Wisconsin, U.S.A.) are shown at the right. The primer pairs used in each set of the amplification reactions were as follows: ITS-1T/ITS-4T for amplifying the nuclear-encoded ribosomal RNA (Kroken & Taylor, 2000), 23S-AL1/23S-AL2 and psbAchl1/psbAchl2 for amplifying the chloroplast-encoded *psbA* and LSU rDNA genes, respectively, which were designed in our laboratory. Asterisks indicate the template DNAs extracted from liquid cultures, the rest correspond to algae cultured in agarized media.

were subjected to electrophoresis through 1.5% Seakem™ agarose gels (FMC Bioproducts, Pennsylvania, U.S.A.) and stained with Gel-Red™ Nucleic Acid Gel Stain (Biotium, California, U.S.A.) (see Fig. 1).

To confirm the specificity of the PCR reactions, both strands of the resultant amplicons were sequenced using the BigDye® Terminator Cycle Sequencing Ready Reaction Kit II, and analyzed by automated Multicapillary Electrophoresis on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems Inc., California U.S.A.).

## ■ RESULTS AND DISCUSSION

The obtained sequences (Appendix) were of high quality, and corresponded to the reference sequence, as confirmed by database searches with the Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (Altschul & al., 1997). The results indicated successful and accurate amplification of all DNAs isolated by the new method. Figure 1 shows an agarose gel electrophoresis of several PCR amplification products from a selection of tested organisms. Additional loci were PCR amplified, including chloroplast-specific genes *rbcL*, *psaC* and *petB* (data not shown). DNA isolated with this method was stable for at least four months (storage at 4°C) and allowed amplifying up to 2000 bp.

The tested protocol has proven to be efficient and rapid for isolation of DNA with an average $A_{260}/A_{280}$ ratio of 2.28, which is slightly higher than other assayed protocols (Table 1). The DNA yield was in the range of 5–35 ng per $10^6$ cells. Spectrophotometric measurements were carried out with Nanodrop (Thermo Fisher Scientific Inc, Wilmington, Massachusetts, U.S.A.). It should be noted that this procedure for DNA extraction has worked out with all the tested organisms comprising twenty-two green and non-green algae, two cyanobacteria and seven plants (Appendix). This procedure requires a minimal amount of starting material (only $5 \times 10^6$–$20 \times 10^6$ cells in the case of algal cultures, and 15 mg in the case of plant tissues). It also works well with algal cultures in agarized media, making it suitable for analyzing algal colonies after their isolation from environmental sources. Other advantages of this procedure for DNA extraction, in comparison with similar methods, are: (1) its rapidity, taking about 30 min to be completed. It thus allows a high number of samples to be processed in relatively short time; (2) elimination of mechanical grinding (Table 1), thus preventing the possibility of contaminations. The obtained DNA is suitable for at least two hundred 25 µl PCR amplifications with a variety of assayed primers targeting both nuclear- and organelle-encoded genes. Additionally, this procedure includes less hazardous and expensive chemicals than similar procedures. In spite of some personal protective equipment necessary for handling DMSO (eyeshields, gloves), it is less harmful than other chemical products frequently used in molecular biology (e.g., phenol, β-mercaptoethanol). Furthermore, residual DMSO in DNA preparations not only does not inhibit PCR amplification, but can improve the yield (Winship, 1989; Cheng & al., 1994).

The new DNA extraction method should be helpful for phycologists and lichenologists to examine photobiont isolates by molecular approaches in a faster and easier manner than with currently available techniques. Moreover, since it also works with land plants, it could be useful for all plant scientists who require a quick isolation method when only small amounts of material are available.

**Table 1.** Comparison of different assayed protocols for DNA extraction to evaluate the new DNA extraction method.

| | DMSO | Cenis, 1992 | Doyle & Doyle, 1990 |
|---|---|---|---|
| Protocol step | | | |
| Liquid nitrogen | – | – | + |
| Grinding | – | + | + |
| Extraction buffer | – | + | + |
| DMSO | + | – | – |
| β-mercaptoethanol | – | – | + |
| Phenol-chlorophorm | – | – | + |
| Freezing | – | + | – |
| Efficiency (ng DNA/$10^6$ cell) | 5–35 | 5–96 | 11–14 |
| Purity ($A_{260}/A_{280}$) | 2.28 | 1.97 | 2.12 |
| Time | 30 min | 35 min | 60 min |

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## ■ LITERATURE CITED

**Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J.** 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389–3402.

**Bold, H.C. & Parker, B.C.** 1962. Some supplementary attributes in the classification of *Chlorococcum* species. *Arch. Mikrobiol.* 42: 267–288.

**Cenis, J.L.** 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Res.* 20: 2380.

**Cheng, S., Fockler, C., Barnes, W.M. & Higuchi, R.** 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. U.S.A.* 91: 5695–5699.

**del Campo, E.M., Casano, L.M., Gasulla, F. & Barreno, E.** 2009. Presence of multiple group I introns closely related to bacteria and fungi in plastid 23S rRNAs of lichen-forming *Trebouxia*. *Int. Microbiol.* 12: 59–67.

**Doyle, J.J. & Doyle, J.L.** 1990. Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15.

**Fawley, M.W. & Fawley, K.P.** 2004. A simple and rapid technique for the isolation of DNA from microalgae. *J. Phycol.* 40: 223–225.

**Friedl, T.** 1995. Inferring taxonomic positions and testing genus level

assignments in coccoid green lichen algae: A phylogenetic analysis of 18S ribosomal RNA sequences from *Dictyochloropsis reticulata* and from members of the genus *Myrmecia* (Chlorophyta, Trebouxiophyceae cl. nov.). *J. Phycol.* 31: 632–639.

**Hebert, P.D.N. & Gregory, T.R.** 2005. The promise of DNA barcoding for taxonomy *Syst. Biol.* 54: 852–859.

**Huss, V. A., Ciniglia, C., Cennamo, P., Cozzolino, S., Pinto, G. & Pollio, A.** 2002. Phylogenetic relationships and taxonomic position of *Chlorella*-like isolates from low pH environments (pH < 3.0). *BMC Evol. Biol.* 2: 13.

**Khan, H., Parks, N., Kozera, C., Curtis, B.A., Parsons, B.J., Bowman, S. & Archibald, J.M.** 2007. Plastid genome sequence of the cryptophyte alga *Rhodomonas salina* CCMP1319: Lateral transfer of putative DNA replication machinery and a test of chromist plastid phylogeny. *Molec. Biol. Evol.* 24: 1832–1842.

**König, J. & Peveling, E.** 1984. Cell walls of the phycobionts *Trebouxia* and *Pseudotrebouxia*: Constituents and their localization. *Lichenologist* 16: 129–144.

**Krienitz, L., Hegewald, E., Hepperle, D. & Wolf, M.** 2003. The systematics of coccoid green algae: 18S rRNA gene sequence data versus morphology. *Biologia* 58: 437–446.

**Krienitz, L., Hegewald, E.H., Hepperle, D., Huss, V.A.R., Rohrs, T. & Wolf, M.** 2004. Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). *Phycologia* 43: 529–542.

**Kroken, S. & Taylor, W.J.** 2000. Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. *Bryologist* 103: 645–660.

**López-Bautista, J.M., Rindi, F. & Guiry, M.D.** 2006. Molecular systematics of the subaerial green algal order Trentepohliales: an assessment based on morphological and molecular data. *Int. J. Syst. Evol. Microbiol.* 56: 1709–1715.

**Nikolopoulos, D., Korgiopoulou, C., Mavropoulos, K., Liakopoulos, G. & Karabourniotis, G.** 2008. Leaf anatomy affects the extraction of photosynthetic pigments by DMSO. *Talanta* 76: 1265–1268.

**Reynolds, M.M. & Williams, C.G.** 2004. Extracting DNA from submerged pine wood. *Genome* 47: 994–997.

**Winship, P.R.** 1989. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucleic Acids Res.* 17: 1266.

**Yoon, H.S., Müller, K.M., Sheath, R.G., Ott, F.D. & Bhattacharya, D.** 2006. Defining the major lineages of red algae (Rhodophyta). *J. Phycol.* 42: 482–492.

**Appendix.** Taxon; species; strain/locality; GenBank accession number.

**Chlorophyta – Chlorophyceae;** *Pseudochlorella aquatica*; SAG 2149; GQ168949. *Stichococcus bacillaris*; SAG 335-3; GQ168950. *Trochiscia gutwinskii*; SAG 102.80; GQ168953. *Trochiscia hystrix*; SAG 103.80; GQ168954. **Chlorophyta – Trebouxiophyceae;** *Elliptochloris bilobata*; SAG 245.80; GQ168945. *Elliptochloris reniformis*; SAG 2200; GQ168955. *Leptosira obovata*; SAG 445-1; GQ168956. *Myrmecia bisecta*; SAG 2043; GQ168957. *Nannochloris atomus*; SAG 14.87; GQ168947. *Trebouxia angustilobata*; SAG 2204; FJ626724. *Trebouxia arboricola*; SAG 219-1A; FJ626725. *Trebouxia asymmetrica*; SAG 48.88; AJ249565. *Trebouxia australis*; SAG 2205; FJ626726. *Trebouxia brindabellae*; SAG 2206; FJ626727. *Trebouxia decolorans*; UTEX B781; FJ626728. *Trebouxia erici*; SAG 32.85; FJ626729. *Trebouxia gelatinosa*; UTEX906; FJ626730. *Trebouxia glomerata*; SAG 100.80; FJ626731. *Trebouxia irregularis*; SAG 33.85; FJ626732. *Trebouxia jamesii*; UTEX 2233; FJ626733. *Trebouxia showmanii*; SAG 2009; FJ626734. *Trebouxia simplex*; SAG 101.80; FJ626735. *Trebouxia suecica*; SAG 2207; FJ626736. *Trebouxia sp.*; axenic culture of this alga after its isolation from the lichen *R. farinacea*, stored in the Department of Botany of the Universitat de Valencia (Valencia – Spain) and Department of Plant Biology of the Alcalá de Henares University (Madrid – Spain); FJ418565. **Chlorophyta – Prasinophyceae;** *Tetraselmis chui*; SAG 8-6; GQ921943. **Chlorophyta – Ulvophyceae;** *Trentepohlia annulata*; SAG 20.94; GQ168951. *Trentepohlia spec.*; SAG 118.80; GQ168952. **Streptophyta;** *Porella platiphylla*; Cercedilla (Madrid-Spain); GQ916676. *Equisetum palustre*; "Henares River Valley" (Guadalajara-Spain); GQ916689. *Malva sylvestris*; "Henares River Valley" (Guadalajara-Spain); GQ916664. *Petroselinum crispum*; nursery-garden; GQ916668. *Retama sphaerocarpa*; "Henares River Valley" (Guadalajara-Spain); GQ916670. *Saintpaulia ionantha*; nursery-garden; GQ916671. *Valerianella locusta*; nursery-garden; GQ916672. **Heterokontophyta – Xanthophyeae;** *Heterococcus brevicellularis*; SAG 835-1; GQ168958. *Heterococcus mainxii*; SAG 835-6; GQ168946. **Cyanobacteria;** *Nostoc insulare*; SAG 54.79; GQ168959. *Nostoc ellipsosporum*; SAG 1453-7; GQ168948.