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ENCAPSULATION DEHYDRATION COLLAGITIVE CRYOPROTECTIVE STRATEGIES AND AMPLIFIED FRAGMENT LENGTH POLYMORPHISM MARKERS TO VERIFY THE IDENTITY AND GENETIC STABILITY OF EUGLENIOIDS FOLLOWING CRYOPRESERVATION

Keith Harding¹*, Julia Müller², Hella Timmermann², Maike Lorenz², John G. Day³ and Thomas Friedl²

¹Plant Conservation Group, University of Abertay Dundee (*current address: Damar Research Scientists, Damar, Drum Road, Cupar, Fife, KY15 5RJ, UK)
²Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Abteilung Experimentelle Phykologie und Sammlung von Algenkulturen (SAG), Universität Göttingen, Untere Karspüle 2, 37073 Göttingen, Germany
³Culture Collection of Algae and Protozoa (CCAP), Scottish Association for Marine Science, Scottish Marine Institute, Dunbeg, Argyll, PA37 1QA, UK

Abstract

An encapsulation/dehydration procedure was developed for Euglena gracilis Klebs as a ‘model alga’ to examine various cryoprotective regimes combined with controlled rate cooling to cryopreserve other Euglenoid taxa. Cryoprotective variables were optimised to enable reproducible growth following a combination of alginate encapsulation, sucrose osmotic dehydration, air desiccation, methanol treatment, cooling to -40°C and plunging into liquid nitrogen (LN). Amplified Fragment Length Polymorphism (AFLP) analysis was adapted to: (i) verify algal identity by discriminating between different Euglenoids and (ii) examine the genetic stability of algal cultures prior to various stages of cryoprotective treatments and following exposure to LN. AFLPs were highly reproducible (>99%) as reliable diagnostic markers, where a single DNA fragment change accounted for ~0.4% of the detectable variation in an AFLP pattern. AFLP changes were detected in cryoprotective treatments following LN exposure. Successive stages of the dehydration and desiccation treatments did not accumulate AFLP changes indicating these are random events.

Keywords: AFLP; cryopreservation; encapsulation dehydration; genetic stability; microalgae

INTRODUCTION

Cryogenic storage of algal cultures has been accomplished over 40 years (23, 24) by a number of freezing protocols that utilize colligative cryoprotection and controlled rate cooling which vary from species to species (33). The ‘traditional’ two-step cooling to transitional temperatures prior to exposure in LN has been applied successfully to a range of taxa including numerous green algal strains (27); diverse eukaryotic algae (2); blue-green
algae/cyanobacteria (4) and marine microalgae (31). Many of the ‘trial and error’ methods (33, 35) have variable levels of success, as many algae remain difficult to freeze (5, 31) or the methods are not robust enough to enable their routine use by the service sector (4, 7). Within a Biological Resource Centre (BRC) algal culture collection (15) the Euglenophyta is of particular scientific importance (8). This group of protists includes pigmented strains, natural achlorophylous taxa and colourless mutants derived from photoautotrophic strains (25). Given the complexity of algal diversity, there has been limited success with the more intricate, freeze-recalcitrant microalgae such as the freshwater Euglenophytes. Typically these have low post-thaw viability levels (13). Earlier studies (24, 28) report the regrowth of *Euglena gracilis* cultures following two-step cooling but for these ‘freeze-recalcitrant’ algae alternative cryopreservation strategies are required as they are sensitive to traditional freezing methods possibly due to their structural complexity (7, 8, 14).

Vitrification-based protocols for microalgae (20) have developed alongside ‘ice-free’ cryopreservation approaches in higher plants (3). To achieve the vitrified state requires the progressive removal of water from a specimen (6). Where the viscosity of intracellular solutes rises during cooling there is an inhibition of the molecular mobility of water below the homogeneous ice nucleation (-40°C) temperature (32). At a point where the liquid forms a glass, an amorphous, non-crystalline state defined by the (Tg) glass transition temperature (20). Cryoprotective vitrification using alginate encapsulation/dehydration has been demonstrated to cryopreserve a range of microalgae (22) as well as gametophytes of marine Brown algae (34, 36). Its application has been widely extended to other fresh water algal strains to include: polar chlorococcal isolates (10), diverse terrestrial (21), soil (26) and red microalgae (1). Other cryoprotective strategies (14, 20) have been used to reduce freezing damage to sensitive algae, which integrate the application of alginate encapsulation, osmotic dehydration, desiccation and colligative cryoprotection with controlled rate cooling (7, 26, 34, 36) but they appear to be limited in their application to algal collections. These combined cryoprotective approaches may overcome the obstacles that restrict their more general use to cryopreserve a wider range of strains within collections.

The use of suboptimal freezing protocols affects the regrowth of viable cells following LN exposure (14, 29) and under such conditions may alter some phenotypic and genotypic characteristics from the original culture (9). Though assurance of stability was shown in strains of the cryopreservation tolerant *Chlorella vulgaris* Beijerinck strains (30), a study of 27 eukaryotic freshwater and terrestrial microalgae using a non-optimised freezing protocol reported differing levels of survival and stability (29). The present study aims to: (i) assess the application of combined encapsulation/dehydration, colligative cryoprotection and controlled rate cooling strategies in various strains of the Euglenoids and (ii) evaluate the use of AFLP markers to verify Euglenoid identity and genetic stability of the strains following cryostorage.

**MATERIAL & METHODS**

**Euglenoid strains and culture conditions**

*Astasia longa* E.G.Pringsheim SAG 1204-17a (type strain), *A. longa* SAG 1204-17b, *Colacium sideropus* Skuja SAG 14.90, *C. sideropus* SAG 1211-1, *Euglena geniculata* Schmitz SAG 1224-4b, *E. gracilis* SAG 1224-5/25 and its colourless heat-induced mutant *E. gracilis* SAG 1224-5/25f were provided by the Sammlung von Algenkulturen (SAG), and *E. gracilis* CCAP 1224/SZ by the Culture Collection of Algae and Protozoa (CCAP). All Euglenoid strains were cultured in appropriate liquid media as recommended on line (www.epsag.uni-goettingen.de and www.ccap.ac.uk). Strains were grown under 14:10 h...
light:dark regime at 20°C with a Photosynthetic Photon Flux Density (PPFD) of 50 µmol photons m$^{-2}$ s$^{-1}$ or at 20°C with a 12:12 h light [PPFD 25 µmol photons m$^{-2}$ s$^{-1}$] dark cycle.

Alginate encapsulation, osmotic dehydration & evaporative (airflow) desiccation

As described previously (6) liquid cultures were centrifuged at low speed (1,000 rpm for 1-2 min.) the supernatant removed and the cells mixed thoroughly with sodium alginate (5% w/v in EG:JM for E. gracilis, or ESP medium for the SAG strains containing 0.5 M sucrose to a cell density of 10$^6$ cells ml$^{-1}$ and then dispensed drop-wise into 150 ml of 100mM CaCl$_2$ where they were maintained for 30-60 min. The CaCl$_2$ solution was removed and the encapsulated algae (denoted as Ca$^{2+}$) osmotically dehydrated in two-steps (48 h in total): firstly with 0.5 M sucrose in culture medium for 24 h (0.5 M) which was replaced by 0.75 M sucrose in culture medium for another 24 h (0.75 M). Encapsulated algal cells (beads) were retrieved from the sucrose medium and excess liquid removed with a sterile filter paper on Petri dishes. Beads were transferred to sterile (open) Petri dishes (9 cm) in an airflow (AF) cabinet and air-dried at 20°C (~50% relative humidity) in the laminar airflow (1 m s$^{-1}$) for 1 to 4 h (AF1-4h) and transferred into cryovials (2.0 ml Nunc) before being cryopreserved.

Residual moisture content

Moisture content was determined from the fresh weight (F.wt.) of a sample of beads (n = 20-25) from each stage in the encapsulation procedure. Beads were dried over silica gel at 105°C overnight to determine their dry weight (D.wt.); the % residual moisture (%RM) per treatment was calculated from these parameters [F.wt. – D.wt./F.wt. x 100 = %RM].

Cryopreservation

Method 1: Cryovials contained 10 samples of (i) beads from Ca$^{2+}$ alginate encapsulated cells, (ii) beads osmotically dehydrated in 0.5 M sucrose for 24 h; (iii) beads following 48 h osmotic dehydration successively in 0.5 M and 0.75 M sucrose (iv) beads after 48 h osmotic dehydration in step with successive 1, 2, 3 and 4 h air desiccation treatments or to ca. 25%RM. These seven separate samples (see Table 1) were transferred to a programmable freezer (Kryo 10, series II, Planar, UK) held at 20°C, then subjected to controlled rate cooling (CRC) at 1°C min$^{-1}$ to 0°C then at 0.5°C min$^{-1}$ to terminal temperatures −40°C or −60°C (held for 15 min) plunged into LN, held for 1 h and rewarmed in a heated water bath at +40°C for ~2 min and cultured for 7 days. Control samples were the same samples without exposure to LN.

Method 2: Duplicate cryovials containing the same (7 pretreatments) samples were treated with 10% (v/v) methanol (MeOH) in culture medium for 15 min at 20°C, subjected to the same controlled rate cooling regimes from 20°C to -40°C or -60°C and held for 15 min then LN plunged for 1 h and rewarmed as above. Control samples were similarly treated but without exposure to LN.

Recovery assessment

Following the seven pretreatments and exposure to LN, rewarmed beads were re-hydrated in liquid medium for 1 h and then cultured in fresh liquid medium. Initially, cultures were maintained in the dark for 3 days and subsequently transferred to standard light and temperature conditions. After 7 days from rewarmed samples, viability assessments were made to determine algal regrowth within the alginate bead and motile cells in liquid medium. Cell counts (n) expressed as n x 10$^4$ cells ml$^{-1}$ were determined using a standard haemocytometer. Statistical analysis was performed using Minitab software.
DNA extraction

Aliquots (1 ml) of algal cultures were centrifuged (1000 x g for 2 min) in 2 ml screw cap microcentrifuge vials (SARSTEDT Ltd., UK). Algal cells were re-suspended in pre-chilled (1ml) extraction solution (Invitek GmbH, Germany) with 5-10 mg glass beads (600 µM) and mechanically disrupted by shaking (5000 rpm) for 30 s in a Mini beadbeater-1 (www.biospec.com). Genomic DNA was extracted twice from each strain using the Invisorb Spin Plant Mini Kit (Invitek GmbH, Germany) following the manufacturers’ instructions (30). DNA concentration was fluorometrically determined (Hoefer, Germany) and high molecular weight DNA samples corresponding to the >20 kb fragment of a lambda DNA standard determined by gel electrophoresis (17) were selected for AFLP analysis.

AFLP reaction conditions

Genomic DNA samples were digested simultaneously by two restriction enzymes EcoRI and MseI and ligated (R-L) in a single reaction as follows (30): 50 ng (5.5 µL) of DNA were incubated with 5 U EcoRI, 1 U MseI and 1 U of T4 DNA ligase (New England Biolabs) in ligase buffer with 55 mM NaCl, 0.55 µg bovine serum albumin, EcoRI and MseI-adapters 5 and 50 pmol respectively in a total volume of 11.0 µL for 3 h at 37°C. Complete DNA digestion was confirmed by agarose gel electrophoresis (17). The R-L reactions were diluted five-fold with sterile distilled water (SDW) and 4 µL of each dilution was used in the preselective amplification with 2.5 pmol of each primers MseI+0 and EcoRI+0, with Taq Polymerase (0.5 U Silverstar, Eurogentec, Belgium) in (10x) reaction buffer with 1.5 mM MgCl₂ and 0.2 mM for each dNTPs (Hybaid, Germany) in a total volume of 20 µL. The amplification cycle was 3 min at 94°C followed by 20 cycles of 20 s at 94°C, 30 s at 56°C and 120 s at 72°C (30). Preselective amplifications products was confirmed by gel electrophoresis, then diluted twenty-fold with SDW prior to the selective amplification. Selective amplification reactions were performed with 4 µL of the diluted preselective amplification mixture as a template for the primer combinations comprising (i) EcoRI+A (7 pmol); (ii) EcoRI+C (5 pmol) and (iii) EcoRI+G (5 pmol) labeled with 6-FAM (blue), VIC (green), and NED (yellow) fluorochromes respectively (Applied Biosystems, Foster City, California, USA) combined with the MseI+C (10 pmol) primer in a total of 20 µL reaction volume. Selective amplification cycles included: 3 min at 94°C, 10 cycles of 20 s at 94°C, 30 s at 65°C except with a decrease of 1°C after each cycle down to the annealing temperature 56°C and 120 s at 72°C followed by 20 cycles of 20 s at 94°C, 30 s at 56°C and 120 s at 72°C (30). AFLP reactions were repeated with two independent DNA extractions from the same algal strain and with two or more replicate reactions for each strain.

Detection of AFLP changes

Fluorescent labelled AFLP fragments were separated by capillary electrophoresis in an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, California, USA). Sizing of the AFLP fragments between 35 and 500 nucleotides was performed with the GeneScan-500 [LIZ] standard (Applied Biosystems) and program GeneScan software (Applied Biosystems). Detectable fragments were evaluated by GenScanner program [provided by D. Hepperle (30)] and AFLP patterns were compared with GelQuest®software (SequentiX - Digital DNA Processing, Klein Raden, Germany). Several primers were tested with DNA extracted from the E. gracilis CCAP 1224/5Z, only the combinations of EcoRI+A/MseI+C, EcoRI+C/MseI+C and EcoRI+G/MseI+C gave satisfactory AFLP patterns with a readable number of reproducible fragments with peaks between 50 to 500 relative fluorescent units (RFUs). The blue fluorochrome (6-FAM) EcoRI+A primer produced 282 fragments; EcoRI+C labeled with the green fluorochrome (VIC) produced 256 fragments and
EcoRI+G with the yellow fluorochrome (NED) gave 249 fragments between 50 to 500 nucleotides.

**Evaluation of AFLP changes**

The extent of background noise and level of baseline variation was determined between both pre- and post-cryopreservation AFLP patterns according to the analytical rigour established by Müller et al. (29, 30). Replicated pre-cryopreservation patterns revealed anomalous peaks at the beginning of the ABI-AFLP fragment separation, as background noise which were excluded from post-cryopreservation comparisons. Other unique non-reproducible fragments (PCR anomalies) were either close to the baseline (0 RFU) or above the baseline threshold of 50 RFUs. These were randomly distributed across the 15 replicated pre-cryopreservation (reference strain) AFLP patterns (see below). All Euglenoid AFLP patterns were critically examined and adjustments were made to the AFLP calculations to eliminate the relative contributions of the baseline and background noise as experimental artefacts (29, 30).

Post-cryopreservation patterns were visually compared to their corresponding reference pattern from the pre-cryopreservation AFLP reactions for the presence or absence of AFLP fragments. All AFLP patterns using identical vertical scales of 0-500 RFUs (y-axis) were visually compared and selected; 50-250 nucleotide profiles were transferred into Powerpoint (Microsoft.com). Reproducibility between AFLP patterns was confirmed by several ‘replicate reactions’ for each algal strain. To determine the extent of PCR anomalies between replicates, each typically consisted of two independent reactions derived from several different DNA extractions. When AFLP changes were detected between replicates these were denoted as ‘variant’ patterns. These AFLP changes represented either missing fragments or de novo peaks typically between 50-100 RFUs. These new fragments often occurred at different nucleotide positions. They were rarely identical between reactions derived from the same DNA extractions or different DNA extractions from the same strain and different Euglenoids. Occasional minor variation in fluorescence intensity (RFU) corresponding to peak heights was observed between different DNA extractions of the same algal strain.

**Calculation of AFLP changes**

Reproducibility of the AFLP procedure was determined using the *E. gracilis* CCAP 1224/5Z as a reference strain by examining the collective number of AFLP changes per total number of detectable AFLP fragments (see above). For example, the level of variation (% AFLP change) for a single nucleotide fragment change within a given AFLP pattern was determined as follows for EcoRI+A primer (1/282 fragments x 100) = 0.35%; EcoRI+C primer (1/256 fragments x 100) = 0.39% and EcoRI+G primer (1/249 fragments x 100) = 0.40%. These values approximate to a single DNA fragment change accounting for ~0.4% of the detectable variation in a single ‘variant’ AFLP pattern. For example, each primer detected the following AFLP changes in several ‘variants’ from a total of 15 AFLP patterns:

i. Primer EcoRI+A detected a single change (1/282 fragments) in 1 variant pattern per 5 analysed AFLP (5 x 282 = 1410 fragments) patterns producing (1/1410 x 100 = ) 0.07% variation per AFLP analysis;

ii. Primer EcoRI+C detected 10 changes (10/256 fragments) in 4 variant patterns per 5 analysed AFLP (5 x 256 = 1280 fragments) patterns producing (10/1280 x 100 = ) 0.78% variation per AFLP analysis;

iii. Primer EcoRI+G detected a single change (1/249 fragments) in 1 variant pattern per 5 analysed AFLP (5 x 249 = 1245 fragments) patterns producing (1/1245 x 100 = ) 0.08% variation per AFLP analysis.
Therefore, the collective (1+10+1 above) number of 12 AFLP changes per total number of (1410+1280+1245) 3935 detectable AFLP fragments produces a level of (12/3935 x 100 =) 0.3% AFLP variation resulting from 6 variants per total of 15 analysed AFLP patterns (see Table 2). The AFLP patterns were >99.7% reproducible as diagnostic markers; the remaining 0.3% variation was attributed to anomalous experimental artefacts.

Similarly, the pre-cryopreservation Euglenoid summary data represents the average number AFLP fragment changes derived from the number of analysed AFLP patterns (Table 2). The data for each cryoprotective stage: alginate encapsulation (Ca²⁺); sucrose osmotic dehydration (0.5 M, 0.75 M) and desiccation (AF1-4 h) and those treatments exposed to LN are represented as an average of the number of AFLP changes derived from the total number of fragments of the analysed AFLP patterns per treatment. The accumulative total variation in AFLP changes for the cryoprotective and cryopreserved treatments represent all the individual stages of the protocol.

RESULTS

Cryopreservation of alginate encapsulated E. gracilis

Table 1 shows the viability of E. gracilis (as the number of free, motile cells within the medium) after 7 days of culture following successive cryoprotective and cryopreservation treatments in combination with a colligative cryoprotectant (MeOH) and controlled rate cooling (CRC) to different terminal transfer temperatures (-40 and -60°C). In the absence of MeOH higher levels of survival were attained in non-cryopreserved controls following osmotic dehydration, desiccation and cooling to -40°C compared to those exposed to -60°C. These samples contrast with the corresponding cryopreserved samples exposed to LN, where all cryoprotective conditions were lethal except for one result at -60°C. The addition of MeOH as a colligative cryoprotective agent improved survival after exposure to LN following transfer from both terminal temperatures (p<0.001). This beneficial effect of MeOH was obvious across all cryoprotective treatments at -40°C. In contrast, recovery was only supported after osmotic dehydration in 0.75 M sucrose and evaporative desiccation for those transferred from -60°C to LN. Notably, the viability was greater following LN treatment compared to their corresponding controls. Statistical analysis indicated there were no significant differences between the cryoprotective treatments with CRC at -40°C or -60°C, there was a significant effect with MeOH following LN exposure.

Table 1. Viability of E. gracilis CCAP 1224/5Z following cryoprotective treatments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CRC-40°C</th>
<th>CRC-40°C+MeOH</th>
<th>CRC-60°C</th>
<th>CRC-60°C+MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cairo²⁷</td>
<td>Control¹</td>
<td>Cryo²</td>
<td>Control¹</td>
<td>Cryo²</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>&gt;200</td>
</tr>
<tr>
<td>0.5 M</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>&gt;200</td>
</tr>
<tr>
<td>0.75 M</td>
<td>4</td>
<td>0</td>
<td>8</td>
<td>&gt;200</td>
</tr>
<tr>
<td>AF1h</td>
<td>5</td>
<td>0</td>
<td>5.5</td>
<td>&gt;200</td>
</tr>
<tr>
<td>AF2h</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>&gt;200</td>
</tr>
<tr>
<td>AF3h</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>&gt;200</td>
</tr>
<tr>
<td>AF4h</td>
<td>4</td>
<td>0</td>
<td>0.2</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

¹non-cryopreserved, cryoprotected controls; ²cryopreserved treatments exposed to LN (viability = number of free cells x 10⁴ ml⁻¹); ³cryopreserved treatments with significant differences (p<0.001) compared to controls.
Cryopreservation of other Euglenoid strains

The Euglenoid group comprise morphologically diverse green pigmented strains, natural achlorophyulous taxa or colourless mutants (Figure 1). All encapsulated strains were cryopreserved following osmotic dehydration and evaporative desiccation to ca. 25%RM content. These cryoprotected beads were treated with MeOH before controlled rate cooling to a terminal transfer temperature of –40°C followed by LN exposure, rewarming, re-hydration and culture for 7 days with an initial 3 day dark phase (Table 2). Cell regrowth was not observed for the A. longa strains or the colourless mutant E. gracilis SAG 1224-5/25f. Bright green cells within the alginate beads were initially observed in E. geniculata and C. sideropus but these bleached following 7 days of culture. Abundantly free, motile cells were observed in E. gracilis CCAP 1224/5Z and SAG 1224-5/25 post LN cultures.

Figure 1. Micrographs of Euglenoid cells: (a) A. longa SAG 1204-17a (colourless); (b) A. longa SAG 1204-17b (colourless); (c) C. sideropus SAG 14.90 (green); (d) C. sideropus SAG 1211-1 (green); (e) E. geniculata SAG 1224-4b (green); (f) E. gracilis CCAP 1224/5Z (green); (g) E. gracilis SAG 1224-5/25 (green); (h) E. gracilis SAG 1224-5/25f colourless mutant.
**Figure 2.** AFLP analysis of DNA extracted from Euglenoid cultures: (a) *A. longa* SAG 1204-17a; (b) *A. longa* SAG 1204-17b; (c) *C. sideropus* SAG 14.90; (d) *C. sideropus* SAG 1211-1; (e) *E. geniculata* SAG 1224-4b; (f) *E. gracilis* CCAP 1224/5Z; (g) *E. gracilis* SAG 1224-5/25; (h) *E. gracilis* SAG 1224-5/25f. (x-axis, the size of fragment in nucleotides; y-axis, RFUs).

**Development of AFLP markers for Euglenoid identification**

Figure 2 shows the characteristic AFLP patterns generated by the (EcoRI+C/Msel+C) primer combination; other primer combinations gave similar AFLP profiles. An interspecific comparison of these Euglenoids demonstrates obvious AFLP marker differences between the colourless *A. longa* (a, b), the green *C. sideropus* (c, d), *E. geniculata* (e) and *E. gracilis* strains (f, g, h). These ALFP markers discriminate between the two strains of *C. sideropus* with fewer differences between *A. longa* and *E. gracilis* strains. AFLP profile comparisons between different strains of *E. gracilis* (f, g, h) is shown by the box marked regions in Figure 2, where AFLP reproducibility was >99% between DNA extractions from the duplicate (reference) CCAP 1224/5Z and the SAG 1224-5/25 strains (Table 2). A single AFLP fragment change (see Materials and Methods) for these strains approximates to <0.4% of the detectable variation in a total number of 3935 and 1614 fragments respectively. Table 2 shows the limits of variability (with 1-6 variant AFLP patterns per 2-15 total AFLP patterns analysed) ranging from 0.1 to 1.7% for these AFLP changes between different DNA extractions, AFLP reactions and Euglenoid strains.

**Table 2. Viability and pre-cryopreservation AFLP analysis of Euglenoid algae**

<table>
<thead>
<tr>
<th>Alga</th>
<th>Strain</th>
<th>Pigment</th>
<th>Viability</th>
<th>Variants/total</th>
<th>% AFLP variation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. longa</em></td>
<td>SAG 1204-17a</td>
<td>Colourless</td>
<td>-</td>
<td>4/6</td>
<td>0.9 (14/1614)</td>
</tr>
<tr>
<td><em>A. longa</em></td>
<td>SAG 1204-17b</td>
<td>Colourless</td>
<td>-</td>
<td>1/6</td>
<td>0.1 (2/1614)</td>
</tr>
<tr>
<td><em>C. sideropus</em></td>
<td>SAG 14.90</td>
<td>Green (+)</td>
<td>1/2</td>
<td>0.4 (2/538)</td>
<td></td>
</tr>
<tr>
<td><em>C. sideropus</em></td>
<td>SAG 1211-1</td>
<td>Green (+)</td>
<td>2/4</td>
<td>0.8 (9/1076)</td>
<td></td>
</tr>
<tr>
<td><em>E. geniculata</em></td>
<td>SAG 1224-4b</td>
<td>Green (+)</td>
<td>6/15</td>
<td>0.3 (12/3935)</td>
<td></td>
</tr>
<tr>
<td><em>E. gracilis</em></td>
<td>CCAP 1224/5Z</td>
<td>Green +++</td>
<td>4/6</td>
<td>0.3 (5/1614)</td>
<td></td>
</tr>
<tr>
<td><em>E. gracilis</em></td>
<td>SAG 1224-5/25</td>
<td>Green +++</td>
<td>4/4</td>
<td>1.7 (18/1076)</td>
<td></td>
</tr>
</tbody>
</table>

1Viability as none (-); initially positive in the beads (+) and high level of survival in the beads with active regrowth of free cells (+++)
2Number of variant AFLP patterns per total number of AFLP patterns analysed
3Data represents the % mean AFLP variation for the presence/absence of DNA fragments and in parentheses the collective number of AFLP changes per total number of AFLP fragments.

**Genetic stability AFLP assessments**

The same AFLP markers were used to assess the genetic stability of *E. gracilis* (1224-5/5Z) cultures. Figure 3 compares the AFLP patterns of the *E. gracilis* reference (1224-5/5Z) strain with a cryopreserved *E. gracilis* culture (following encapsulation, osmotic dehydration, 4 h desiccation, MeOH treatment, controlled rate cooling to -40°C and LN exposure). The AFLP fragment distribution in the AFLP pattern for the cryopreserved *E. gracilis* culture is representative of the other cryoprotective treatments. There were several detectable AFLP fragment differences between the reference strain and cultures following alginate encapsulation cryoprotective treatments before (-LN) and after (+LN) plunging into LN.

Table 3 shows the AFLP analysis of 6 cryoprotective (-LN) treatments without MeOH treatment following controlled rate cooling to -40°C before plunging into LN. AFLP marker
variability between the treatments within the protocol ranged from 0.1 to 0.9% resulting from 1-4 variant AFLP patterns per 2-6 total AFLP patterns analysed. Overall, there was a total collective variation of 2.5% (21/6456) across all 6 cryoprotective treatments which produced on average an AFLP change of 0.4% per treatment. Of the total 21 AFLP changes 42.9% represented missing fragments and 57.1% were new fragments in the AFLP patterns. The AFLP changes for the seven cryopreserved (+LN) samples varied from 0 to 1.4% in 1-3 variant AFLP patterns per 2-4 total AFLP patterns analysed. Overall, a total collective variation of 3.6% (37/6994) across all the treatments produced an average AFLP change of 0.5% per treatment. Of the 37 AFLP changes observed 86.5% were missing fragments with 13.5% new fragments in the AFLP patterns. AFLP fragment changes were rarely observed in the same positions within patterns across all -LN and +LN treatments; these values were not statistically significant between treatments (Table 3).

**Figure 3.** AFLP analysis of *E. gracilis* (CCAP 1224/5Z) reference strain and the cryopreserved *E. gracilis* culture following encapsulation, osmotic dehydration, 4 h desiccation, methanol treatment, control rate cooling and plunging into LN

**Table 3.** AFLP variation in *E. gracilis* CCAP 1224/5Z cultures after alginate encapsulation cryoprotective treatments before (-LN) and after (+LN) plunging into LN

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<thead>
<tr>
<th>Treatments</th>
<th>Variants/total&lt;sup&gt;1&lt;/sup&gt;</th>
<th>% AFLP &lt;sup&gt;-LN&lt;/sup&gt;&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Variants/total&lt;sup&gt;1&lt;/sup&gt;</th>
<th>% AFLP &lt;sup&gt;+LN&lt;/sup&gt;&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; alginate</td>
<td>-</td>
<td>-</td>
<td>3/4</td>
<td>1.4 (15/1076)</td>
</tr>
<tr>
<td>0.5 M sucrose</td>
<td>3/6</td>
<td>0.2 (3/1624)</td>
<td>1/2</td>
<td>0.4 (2/538)</td>
</tr>
<tr>
<td>0.75 M sucrose</td>
<td>2/2</td>
<td>0.9 (5/538)</td>
<td>0/4</td>
<td>0.0 (0/1076)</td>
</tr>
<tr>
<td>AF&lt;sub&gt;1h&lt;/sub&gt;</td>
<td>2/2</td>
<td>0.4 (2/538)</td>
<td>3/4</td>
<td>1.0 (11/1076)</td>
</tr>
<tr>
<td>AF&lt;sub&gt;2h&lt;/sub&gt;</td>
<td>2/6</td>
<td>0.1 (2/1614)</td>
<td>2/4</td>
<td>0.8 (9/1076)</td>
</tr>
<tr>
<td>AF&lt;sub&gt;3h&lt;/sub&gt;</td>
<td>1/2</td>
<td>0.6 (3/538)</td>
<td>0/4</td>
<td>0.0 (0/1076)</td>
</tr>
<tr>
<td>AF&lt;sub&gt;4h&lt;/sub&gt;</td>
<td>4/6</td>
<td>0.4 (6/1614)</td>
<td>0/4</td>
<td>0.0 (0/1076)</td>
</tr>
<tr>
<td>Total</td>
<td>14/24</td>
<td>2.5 (21/6456)</td>
<td>9/26</td>
<td>3.6 (37/6994)</td>
</tr>
<tr>
<td>Mean (±SD)</td>
<td>2/4</td>
<td>0.4 (±0.29)</td>
<td>1/4</td>
<td>0.5 (±0.57)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Number of variant AFLP patterns per total number of AFLP patterns analysed  
<sup>2</sup>Data represents the % mean AFLP variation for the presence/absence of nucleotide fragments and in parenthesis the collective number of AFLP changes per total number of detectable AFLP fragments; there were no significant differences between +LN and –LN treatments.

**DISCUSSION**

Effective cryopreservation of algae requires the following criteria: (i) optimisation of the cryogenic state; (ii) retention of biochemical and metabolic functionality and (iii) protection of uniquely specialised anatomical structures. Like many algae (35) *Euglena* is not able to
survive exposure to LN without cryoprotection (11, 14). Encapsulation in alginate or in combination with osmotic dehydration and/or desiccation followed by plunging in LN is usually lethal or with infrequent delayed regrowth. These cryoprotective treatments effectively reduce the moisture content (ca. 25%) to produce a glassy state following LN exposure suggesting that residual water remains to form lethal intracellular ice (20). Its removal from vital intracellular structures appears to be achieved by combining two-step cooling to improve cell survival. Viability is critically dependent on the cooling rate to determine the cryogenic state, where rates $<0.5^\circ\text{C min}^{-1}$ cause excessive dehydration and those $>0.5^\circ\text{C min}^{-1}$ are insufficient to cryoprotect against the lethal effects of intracellular ice (14). Exposing encapsulated cryoprotected E. gracilis cells to a cooling rate of 0.5°C min$^{-1}$ showed marked differences in viability between the -40/-60°C terminal transfer temperatures. The 20°C differential suggests increased cell death (at -60°C) may result from the ‘colligative’ solution effects caused by excessive dehydration (14). Higher viabilities were observed across all encapsulated cryoprotective treatments cooled to -40°C indicating this temperature is a critical factor (13). As a colligative cryoprotectant, the beneficial effects of methanol to algae are well known (35), the relief from deleterious solution effects at -60°C by methanol were observed by the increase in cell viability (Table 1). Inhibitory effects of cell density on viability have been observed in other algae (5). E. gracilis alginate encapsulation at a density of $10^6$ cells ml$^{-1}$ may inhibit growth of the control cultures, in contrast to cell regrowth following cryopreservation which differed by several orders of magnitude. This observation may also be explained by an initial decrease in viable cell numbers following LN exposure thereby reducing cell competition effects and increasing nutrient availability. There are other technical points which critically influence the vitrified state and viability after cryopreservation (7, 11, 13). Notably, in the present study regrowth was achieved following: (i) low speed centrifugation to pellet cell cultures prior to alginate encapsulation; (ii) preloading alginate encapsulated beads with (0.5 M) sucrose; (iii) performing methanol cryoprotection for 15 min at ambient (20°C) temperature; (iv) holding samples at -40°C for 15 min before transfer into LN and (v) directly rewarming samples for $\sim$2 min at 40°C. Improvement to cell viability following encapsulation/dehydration or the combined cryoprotective strategy was not evident using 2-stage rewarming (7, 14).

Applying the combined cryoprotective protocol in the comparative study to other Euglenoid taxa representing physiological diversity (photoauxotrophic/heterotrophic habit) of the flagellate group, some chlorophyllous E. geniculata and C. sideropus strains became bleached and died within the alginate beads, whereas the non-pigmented taxa, either natural or mutant strains failed to survive. Although, the cryogenic conditions support the vitrified state and actively motile cells of E. gracilis other non-cryogenic factors appear to underlie those responses that possibly affect metabolic functionality (18). Sensitivity to the stress-related stages of methanol treatment, chilling, cooling and immersion in LN has been shown to impair photosynthetic capacity (7, 11). Despite the 3-day dark photo-protective recovery period gradual cell death occurred with the progressive loss of photosynthetic pigments. Biochemical dysfunction caused by free radical-mediated damage has been indicated to occur during the preparative cryostorage stages (7, 11). The extent of oxidative damage during cryopreservation to E. gracilis was evident (18) without additional exogenous antioxidant protection (11, 12). Achromophylous Euglenoid taxa may be increasingly sensitive to the individual stages of combined cryoprotection. Colourless Euglenoids have more mitochondria than the equivalent–sized green strains. Also, the green Euglenoid strains decolourised by heat have a seven-fold increase in mitochondrial volume reflecting the metabolic changes from photoauxotrophic to heterotrophic nutrition (25). These mitochondrial attributes coupled with the fact that the formation of two mitochondrial enzymes fumarase and succinate dehydrogenase necessary for dark respiration of substrates are repressed by light (25) may
further explain the observed differential responses between Euglenoid taxa following cryopreservation.

The different cryoprotective mechanisms appear to be complementary in resolving the issues of cryostorage for *E. gracilis*. Although, encapsulation/dehydration is not entirely cryoprotective, the alginate cell matrix appears to provide some structural protection to the cellular fabric and intricate anatomical structures. As freshwater organisms, Euglenoids are sensitive to osmotic changes during two-step colligative cooling causing cell volume changes by dehydration and destabilisation of the membrane structures (14). Alginate entrapment appears to support the structural integrity of Euglena’s elongate cells thereby limiting the effect of the ‘rounding up’ phenomena observed during methanol and cooling treatments (7). The prevention of cell distortion events is likely to reduce rupture of the pellicle and plasma membrane thereby ensuring effective osmoregulation (13). Cell immobilisation may also protect against damage induced by freeze fracture to the flagellum following release from the alginate matrix during recovery after LN exposure. Complex cell structures, such as the contractile vacuole that regulate water status are vital determinants to Euglenoids’ survival. Mechanistically, cell viability possibly improves by the penetrating colligative action of methanol in aiding the removal of residual water from the contractile vacuole and other cellular vesicles. Methanol would further depress the freezing point of solutes thereby reducing intracellular ice formation. It would also, for many algae mitigate the deleterious solution effects from excessive concentration of intracellular solutes caused by dehydration during controlled cooling (5, 35).

AFLPs as a taxonomic marker has proved to be a valuable tool to resolve genetic differences between strains at the molecular level (30) for the identification microalgal strains (29). The discriminatory power of the AFLP technique is also shown in the Euglenoid interspecific study between the achlorophylous and pigmented strains; AFLP patterns show clear differences at the genus and species levels. The ‘proof-reading’ of existing strains is essential to resolving the issues of conspecificity by identifying errors and limiting unwanted redundancy within an algal collection (30). While the problems of selective pressure and/or genetic drift during routine serial subculture cannot fully ensure phenotypic or genotypic stability in the maintenance of strains (9), the use of AFLP markers to authenticate algal strains would also serve as a ‘benchmark’ to establish quality assurance standards in the BRCs provision of organisms and their services (15). The risk of genomic alteration is fundamental to any preservation procedure particularly where protocols are not optimised (29). Many markers are available (16) but the choice of AFLP technique is ideal for microalgae (30) due to their size and lack of phenotypic distinction between individuals of a strain. Euglenoid AFLP markers were applied to assess the genetic stability of surviving strains before and after cryopreservation (19). Vitrification is likely to cause progressive osmotic stress during these cryoprotective stages coupled with colligative damage during controlled cooling. An examination of the individual cryoprotective components of the encapsulation/dehydration protocol showed several detectable AFLP changes. There was no obvious correlation between the AFLP changes with progressive cryoprotection steps, suggesting primer site mutation events are not accumulative with each successive treatment. Changes in the ‘dehydration’ or ‘desiccation’ stages have a largely stochastic distribution with fewer AFLP changes in the later stages following LN exposure signify there are no synergistic interactions or additive effects between treatments.

In conclusion, this study demonstrates the reproducible growth of *E. gracilis* cells following the combined treatments of dehydration, desiccation, methanol, controlled cooling to -40°C and plunging into LN. AFLPs were highly effective as a diagnostic marker to verify algal identity, i.e. discriminate between different Euglenoids and assess genetic stability following cryopreservation.
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REFERENCES


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